BIOLOGICALLY ACTIVE ADDITIVES - RELEVANCE FOR BONE REGENERATION

Adelina S. Plachokova
BIOLOGICALLY ACTIVE ADDITIVES - RELEVANCE FOR BONE REGENERATION

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op donderdag 4 September 2008
om 10:30 uur precies

door

Adelina Stefanova Plachokova
geboren op 19 Juli 1975
te Sofia (Bulgarije)
BIOLOGICALLY ACTIVE ADDITIVES - RELEVANCE FOR BONE REGENERATION

An academic essay in the field of Medical Sciences

Doctoral thesis

to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the rector magnificus, prof. mr. S.C.J.J. Kortmann,
according to the decision of the Council of Deans

to be defended in public on Thursday, 4th of September 2008,
at 10:30 a.m.

by

Adelina Stefanova Plachokova
born on 19th of July 1975
in Sofia (Bulgaria)
Table of Contents

Chapter 1 General Introduction

Chapter 2 The Effect of Platelet-Rich Plasma on Bone Regeneration in Dentistry: a Systematic Review

Chapter 3 The Early Effect of Platelet-Rich Plasma on Bone Healing in Combination with an Osteoconductive Material in Rat Cranial Defects

Chapter 4 The Late Bone Regenerative Effect of Platelet-Rich Plasma in Combination with an Osteoconductive Material in Rat Cranial Defects

Chapter 5 Bone Regenerative Properties of Rat, Goat and Human Platelet-Rich Plasma in Combination with Allograft and Autograft in an Immunodeficient Rat Model

Chapter 6 Bone Regenerative Properties of Injectable PLGA/CaP Composite with TGF-β1 in a Rat Augmentation Model

Chapter 7 The Bone Regenerative Properties of Emdogain® adsorbed onto PLGA/CaP Composites in an Ectopic and Orthotopic Rat Model

Chapter 8 General Discussion

Chapter 9 Summary and Address to the Aims

Chapter 10 Samenvatting en evaluatie van de doelstellingen

Chapter 11 Acknowledgements

Chapter 12 Curriculum Vitae

List of Publications
Chapter 1

General Introduction
1. Background

Currently, millions of people worldwide are affected by musculoskeletal diseases, which often lack optimal treatment, and result in concomitant long-term pain and physical disability. Other health problems of major public and treatment concern include complicated bone fractures and intra-oral bone loss caused by periodontal disease. Unfortunately, conventional treatment methods for bone healing have their limitations and do not always provide a successful solution for the above mentioned problems. Therefore, increasing efforts are focused on the development of novel therapeutic strategies to enhance bone healing and regeneration, and hence, to improve the quality of life for millions of people.

Traditional treatment methods for promoting bone healing primarily utilize bone grafts or synthetic materials to fill bone defects and to provide structural support. Bone autografts are still considered the ‘gold standard’ for treating bone defects due to their osteogenicity and low risk of adverse immune response [1]. Allografts involve harvesting and processing bone from a different individual of the same species, which is subsequently transplanted into the patient. Xenografts, predominantly of bovine or porcine origin, are also used to treat bone defects. Disadvantages of using autografts for transplantations include limited availability of viable bone tissue, donor site morbidity, and need for an additional surgical procedure, whereas the main concern related to the use of allografts and xenografts is the potential disease transmission. In cases where bone grafts from human or animal sources are not feasible (e.g. limited supply or insufficient bone volume available), synthetic graft materials (alloplasts) are used. In comparison with auto- and allografts, the limitations of these synthetic bone-replacement materials comprise poor integration with the surrounding tissue, potential need for future retrieval or replacement, and inability to adapt to the dynamic environment. A guided tissue-regeneration (GTR) approach has offered some additional, beneficial bone healing options, since it employs materials that support natural bone-formation processes (e.g. hydroxyapatite, calcium phosphate, bioactive glasses, collagen, and biodegradable polymers) [2]. Nevertheless, the success of GTR is not always predictable, and this approach is limited in its application mainly to small bone defects, such as those caused by periodontal disease [3,4].

In view of the above mentioned, tissue engineering has emerged as a new therapeutic alternative to promote bone healing. In principle, tissue engineering is based on the understanding of tissue formation and regeneration, and aims to induce new functional tissues, rather than just implant new spare parts [5]. Bone tissue engineering aims to regenerate or repair bone tissue by development of bone graft substitutes, which are comparable or superior to autogenous bone and upon implantation will be replaced by patient’s own tissue. In general, tissue engineering approaches consist of three key elements: (1) matrix – also termed scaffold, combined with (2) cells and/or (3) biologically active molecules (e.g. growth factors, cell adhesion molecules) (Figure 1). According to the combination of key elements, bone tissue engineering approaches can be divided into two classes: (i) cell-based, and (ii) growth factors (GFs)-based, with the addition that many types of matrix/scaffold materials are currently commercially available, which are continuously being optimized to more closely mimic the properties of bone tissue.

Figure 1: Tissue engineering approach

Cell-based bone tissue engineering involves loading of osteogenic cells into the matrix/scaffold. Osteogenic cells have to be harvested, isolated, expanded in vitro to higher passages and loaded/seeded to the scaffolds. From a bone tissue engineering perspective, this approach has evident advantages due to the high osteogenic potential of the cells. However, from a manufacturing and clinical point of view, it has a lot of limitations which make the introduction of such a cell-based product to the clinical practice difficult and expensive. The major disadvantages involve the individual patient basis approach (cells are collected from the patient and transplanted into the same patient), invasive harvesting procedure (especially when bone-marrow mesenchymal stem cells...
are collected), time and labour consuming in vitro cell expansion procedure, and the risk of disease transmission or cell infection (during cell culturing outside the body). All the above mentioned makes this cell-based approach currently not very appealing for standard or routine clinical application.

The alternative, a growth factor-based bone tissue engineering approach, seems to provide an easier and more cost-effective way to induce bone regeneration in patients. It includes the delivery of GFs known to regulate key aspects of bone metabolism directly to the defect sites, which upon release promote bone regeneration. The development of recombinant DNA has enabled the production of large amounts of recombinant GFs (rh-GFs) for therapeutic use and has allowed their extensive investigation. GFs can be delivered alone (direct injection), through gene therapy or incorporated in a scaffold material. Preparation of GFs-loaded scaffolds is relatively simple and safe. Moreover, from a clinical point of view it is a non-invasive approach and has universal application. On the basis of the available knowledge, GF-based bone tissue engineering appears to be the most promising and beneficial strategy for creation of the ideal bone graft substitute.

Bioactivation of surfaces with covalent bound peptides or with nanomechanically linked proteins (i.e. cell adhesion molecules) has appeared as another attractive strategy for bone tissue engineering. It is known that the first event that occurs during contact between a biomaterial (i.e. CaP) and biological fluids, is the adsorption of proteins on its surface. The attachment and adhesion of cells resulting in the future organization of the tissues depends on the nature of this adsorbed layer. Therefore, the tailoring or monitoring of peptide or protein immobilization represents one of the most challenging issues in the field of tissue engineering today.

In view of the current limitations of cell-based bone tissue engineering, only matrix-based and GFs-based approaches are discussed in this thesis. The focus is placed on the development of a dedicated bone substitute using biologically active additives to stimulate bone regeneration.

2. Calcium phosphate (CaP) ceramics

Calcium phosphate (CaP) based ceramics are among the most frequently used materials to facilitate bone healing [6]. Commercially available CaP biomaterials differ in origin (natural or synthetic), composition (hydroxyapatite, beta-tricalcium phosphate, biphasic CaP, etc.), physical form (particulates, blocks, cements, coatings on metal implants, composites with polymers), and physicochemical properties. In general, CaP biomaterials have outstanding properties that make them suitable for clinical application, such as similarity in composition to bone mineral, bioactivity (ability to directly bond to bone tissue), ability to promote cellular function and expression leading to formation of an uniquely strong bone-CaP biomaterial interface, and osteoconductivity. Osteoconduction, as defined by Davies et al. 1998 [7], is the process by which osteogenic cells migrate to the surface of the scaffold through a fibrin clot, which is established right after the material implantation. In addition, CaP biomaterials are reported to be osteoinductive [8]. Osteoinduction is the process by which undifferentiated osteoprogenitor cells are committed to the osteogenic lineage and form osteoprogenitor cells [9]. It has been described that calcium phosphates can play an important role in the process of osteoinduction. Numerous publications have reported already that a wide variety of different CaP ceramics can induce bone formation in various animal models [10-16]. The exact mechanism of osteoinduction by CaP materials is still unknown. It is hypothesized that this material-induced bone formation is the secondary reaction of bone-morphogenetic proteins (BMPs) concentrated in vivo on these materials [11]. On the other hand, the macro- and microstructure as well as chemical composition of the material has also been shown to play an important role in bone induction [17,18].

2.1. Biphasic CaP ceramic (BCP)

Biphasic calcium phosphate (BCP) consists of a mixture of hydroxyapatite (HA; Ca_{10}(PO_4)_6(OH)_2) and beta-tricalcium phosphate (ß-TCP; Ca_3(PO_4)_2) of varying HA/ß-TCP ratios. BCP is obtained by sintering a synthetic or biologic calcium-deficientapatite (Ca/P ratio < 1.67) at a temperature of 700°C and above. Properties of BCP ceramics that define their clinical efficacy and can be modified according to clinical requirements include macroporosity, microporosity, compressive strength, bioactivity (in vitro and in vivo), dissolution, and osteoconductivity [6]. For example, the bioactivity can be controlled by manipulating the composition (HA/ß-TCP ratio), since it is known that ß-TCP has a higher dissolution rate than HA, which has been observed to affect the bone ingrowth [6]. Moreover, BCP can be osteoinductive, since it has a high affinity for proteins, including endogenous BMPs.

Currently, BCP ceramics are recommended for use as an alternative or additive to autogenous bone for orthopedic and dental applications. They are available in the form of particulates, blocks, customized designs for specific applications, and as an injectable biomaterial in a polymer carrier [19]. Commercial biphasic calcium phosphate products include: Triosit® (Biomatlante Ltd, Nantes, France), MBCP® (Biomatlante Ltd), and Osteosynth® (Eincro Biomaterial Co Bella Horizone, Brazil), Straumann® Bone Ceramic (Straumann AG, Basel, Switzerland). Various experimental studies have already demonstrated the potential of BCP ceramic as scaffold for bone tissue engineering [6,20,21].
2.2. Injectable CaP cements
Injectable calcium phosphate (CaP) cements have been developed in the 90’s [22] to overcome disadvantages of prefabricated CaP materials, such as lack of shaping and difficult delivery and maintenance of the prefabricated materials in the defect site. Currently, several injectable CaP cements are commercially available, such as Bone Source® (Stryker Leibinger, Kalamazoo, MI, USA), Norian® (Synthes, Oberdorf, Switzerland), alpha-BSM/Biobon® (Etex Corporation, Cambridge, MA, USA) and Calcibon® (Biomet Merck, Darmstadt, Germany). These cements differ slightly in chemical composition, which results in differences regarding handling properties, setting time, strength and resorbability.

In general, CaP cements are known for their biocompatibility and osteoconductive properties [23]. The major advantage concerning clinical application is that CaP cements can be molded perfectly according to the defect dimensions, after which they set in situ. Injectable CaP cements consist of a powder containing one or more solid compounds of calcium phosphate salts and an aqueous cement liquid. Mixed in an appropriate ratio, a paste is formed which sets at body temperature by entanglement of the crystals precipitating within the paste. After setting, the CaP crystals form a nanoporous structure with pores smaller than 1 μm [22,24]. Although nano- (diameter < 1 μm) and microporosity (diameter < 10 μm) of the CaP cements are favourable for body fluid circulation [25], these do not allow bone ingrowth. Consequently, the ratio of cement surface area to cement volume is low and osteoclastic resorption rate is slow [26], resulting in a slow degradation rate of the CaP cements. In an attempt to overcome these disadvantages, modified CaP cements have been developed [26,27]. Since it has been demonstrated that macroporosity (diameter >100 μm) [25] allows a scaffold to be colonized by osteogenic cells and can increase early degradation, different methods to introduce macroporosity into a CaP cement structure have been studied. For example, to generate macroporosity, NaHCO₃ was added for production of CO₂ gas in the cement [25,26,28]. Although in this way the degradation rate was enhanced and bone ingrowth was achieved, the compressive strength of the macroporous cement was greatly decreased. Another investigated method to generate porosity in the CaP cement involved the incorporation of PLGA microspheres into the scaffold [29] (Figure 2).

Mechanical testing of this composite showed that it is stronger than previously described macroporous cement (i.e. with CO₂ bubbles), but yet weaker than the cement without PLGA microspheres. The major advantage of the incorporation of PLGA microspheres into the CaP cement, besides the induced porosity after microsphere degradation to promote bone ingrowth and enhance CaP degradation, is that PLGA microspheres can be applied for the delivery of biologically active compounds with stimulatory effects on the bone healing process, such as BMP-2 [30]. Finally, the developed PLGA/CaP composite has demonstrated excellent biocompatibility at ectopic and orthotopic implantation sites [29,31].

3. Biologically active additives for bone tissue regeneration
The use of biologically active additives to stimulate bone healing is currently gaining clinical importance.

3.1. Growth Factors
It is known that GFs play an essential role in the healing of injured tissue and act as regulators of the most basic cell functions, using endocrine, paracrine and intracrine mechanisms. Their mode of action is to bind to the extracellular domain of a target growth-factor receptor that, in turn, activates the intracellular signal-transduction pathway [32-34]. These findings have led to a significant research effort aimed at testing different GFs and cytokines as therapeutic molecules for bone tissue repair and regeneration (Figure 3).

![Figure 2: SEM of injectable CaP/PLGA cement (20/80)](image)

![Figure 3: Growth factors for bone regeneration](image)
The most extensively studied bone growth stimulating factors are members of the transforming growth factor-β (TGF-β) superfamily, comprising structurally and functionally related proteins, which regulate key aspects in bone metabolism [35]. This superfamily of molecules includes five TGF-β isoforms (TGF-β 1 to 5), the inhibins/activins, bone morphogenetic proteins (BMPs), and a number of distantly-related molecules [36]. TGF-β is one of the major GFs present in the extracellular matrix of bone tissue. It plays an important role in keeping the balance between the processes of bone resorption and subsequent bone formation. TGF-β1 appears to be the major isoform produced by human osteoblasts. In addition, TGF-β1 is highly expressed during fracture healing [37], suggesting that its role is not restricted to bone development and turnover, but extends to the process of bone repair.

The primary role of TGF-β1 in bone formation seems to be the chemotaxis and mitogenesis of osteoblast precursors. In addition, TGF-β1 affects osteoblast differentiation, matrix formation, and mineralization.

However, there are several disadvantages concerning the application of TGF-β1. Under certain conditions TGF-β1 can also stimulate osteoclast formation and function [38]. Consequently, treatment may induce both bone formation and resorption. Additionally, the half-life of TGF-β1 is too short (~2 min), implying the need for a matrix, which ensures a sustained release of the growth factor. Finally, TGF-β1 is involved in diverse functions outside the bone environment, suggesting that too high levels of TGF-β1 in the circulation or systemic administration of TGF-β1 might cause undesired side effects [39-42].

Despite the shortcomings of TGF-β1, positive effects on fracture healing and bone formation predominate in all in vivo studies. Dosage, application mode and site, follow-up period, and animal species are parameters that influence the outcome of the therapy. An advantage in the use of TGF-β1 is the conservation of the amino acid sequence across species (interspecies homology), ensuring biological activity of recombinant human TGF-β1 in several animal models without facing the problem of antibody responses.

Another naturally occurring type of GFs that stimulate bone formation are BMPs. They were identified and cloned after the discovery that extracts from demineralized human bone matrix induce ectopic bone formation [36]. Currently, more than 20 members of the BMP family have been identified (BMP-2, BMP-4, BMP-7, BMP-8, etc.) [43]. BMP-2 acts primarily as a differentiation factor for bone and cartilage precursor cells during osteogenesis and bone regeneration. BMP-2 and BMP-7 (also known as OP-1) have already been used in clinical trials and have proven their efficacy in the healing of critical-sized fibular defects and tibial non-unions in humans [44].

Other bone growth stimulating factors as present in bone are Insulin-like Growth Factor I and II (IGF-I and IGF-II). IGFs mediate the effects of systemic hormones (e.g. growth hormone), cytokines (e.g. interleukin-1α), and morphogens (e.g. BMPs) in bone formation and healing [45]. Fibroblast Growth Factors (FGFs) also play an important role in bone development and repair. FGFs are autocrine/paracrine regulators of bone formation, which stimulate chemotaxis, proliferation, and matrix synthesis of osteoblasts or osteoblast precursors, but do not directly promote osteoblast differentiation [45].

GFs that stimulate angiogenesis, including Vascular Endothelial Growth Factor (VEGF) and Platelet Derived Growth Factor (PDGF) are also very relevant for bone repair and regeneration. VEGF plays an important role in the regulation of the interaction between osteogenesis and angiogenesis. PDGF is supposed to play a role in the migration of mesenchymal stem cells (MSCs) to the wound healing sites.

The GFs discussed above have been extensively investigated, since the recombinant DNA technology has enabled their availability in large quantities at lower costs. Conventional delivery methods for recombinant GFs in bone tissue engineering employ direct injection of a single GF in the defect site [46]. Due to the short half-life of many recombinant GFs, usually very high doses are required to achieve a therapeutic effect [46,47]. A disadvantage of this delivery approach is that an initial high concentration of the growth factor is provided (burst-release), while maintenance of a much lower level for an appropriate period of time can be much more effective. Such a sustained delivery mimics also better the natural physiological process of bone healing. In addition, under physiological conditions tissue formation and reparative processes rely not only on just one single GF, but on several GFs that work in concert to form functional tissue. To achieve this, an engineering strategy can be followed by designing release systems that are able to deliver low doses of various relevant GFs over the course of several weeks. On the other hand, use can also be made of natural alternative systems, like platelet-rich plasma (PRP).

### 3.2. Platelet-rich plasma (PRP)
Platelet-rich plasma (PRP) is defined as an "autologous concentration of platelets in a small volume of plasma" [48]. It is considered to be a rich source of autologous GFs, due to the many GFs present in the platelets’ alpha-(α-) granules. In vitro studies have demonstrated clearly, that GFs derived from platelets stimulate the proliferation of both human trabecular bone cells [49] and human osteoblast-like cells [50]. The following GFs are reported to be present in platelets α-granules and released upon platelets’ activation: Platelet Derived Growth Factor (PDGF) in its three isoforms (αα, ββ, αβ), Transforming Growth Factor (TGF) in its two isoforms (β1 and β2), Vascular Endothelial Growth Factor (VEGF), Epithelial Growth Factor (EGF), Insulin-like Growth Factor -1 (IGF-1), basic Fibroblast Growth Factor (bFGF), as well as three blood proteins known
to act as cell adhesion molecules for osteoconduction (i.e. fibrin, fibronectin and vitronectin) [51-53]. The active secretion of GFs from platelets begins within 10 min after the regular blood clotting process has started. More than 95% of the presynthesized GFs in the platelets α-granules are secreted within 1 hour (Figure 4).

This release is thought to accentuate the bone reparative response. After the initial burst release of PRP-related GFs, platelets synthesize and secrete additional GFs for the remaining 10 days of their life span. Afterwards, macrophages attracted to the bony wound site take over and secrete additional GFs to regulate and continue bone regeneration.

Briefly, the basic hypothesis of PRP addition to bone grafts is that high concentrations of platelets in a bony wound will increase the local concentration of GFs and subsequently enhance the initial bone healing response. In a later stage, the direct influence of PRP will fade away and physiological mechanisms of bone repair will continue to work on an accelerated level [54].

For bone tissue engineering PRP can be combined with different types of scaffolds, such as mineral and organic bone matrices [52,55]. One potential benefit of this combination is the improved handling and adaptation of the matrix to the injured tissue because the fibrin acts as a biological glue to hold together the matrix particles. Moreover, early vascular invasion is a key factor in bone allograft or xenograft incorporation, which reduces complications related to slow and incomplete integration. For example, Aghaloo et al. evaluated deproteinized bone mineral of bovine origin Bio-oss® (Geistlich, Wolhusen/Switzerland) with and without PRP in rabbit cranial defects and observed significant histomorphometric improvement in bone formation when PRP was applied [56]. Recently, beneficial effects of treating intrabony periodontal defects with PRP combined with different kind of bone substitutes in controlled clinical trials have been reported [56-58]. However, there are controversies in the literature regarding the potential benefits of PRP as an adjunct to osseous regeneration. While some authors have reported significant improvements in tissue healing and bone formation using PRP [53,59,60], others failed to observe improvement [61-63]. So far the best results for bone regeneration are reported when PRP is added to bone grafting material [53]. An explanation can be that only autogenous grafts have receptors to bind GFs released by PRP upon platelets activation [53]. Nevertheless, since favorable bone formation has also been reported when PRP has been combined with bone substitutes, its application together with biomaterials should be further examined and developed. Moreover, it has to be emphasized that upon its first discovery PRP has been directly introduced into the clinic and its exact mechanisms of action still remain unclear.

The feasibility of PRP as scaffold for cell-based bone tissue engineering has also been explored. In most of the approaches mesenchymal stem cells (MSC) have been combined with PRP. MSC-PRP mixture has the advantage of being simultaneously osteogenic, osteoconductive and osteoinductive due to the bone-forming capacity of MSCs and the presence of secreted GFs in the three-dimensional fibrin scaffold. For example, Ito et al. studied the potential of a MSC-PRP mixture compared with autogenous bone, Bio-Oss® and PRP, and found out that this mixture provided greater bone maturation and early-stage bone regeneration compared to the rest of the treatments [64]. Concerning the technique for producing PRP, PRP is derived from the whole blood of the patient through gradient density centrifugation. The technique involves use of a medical cell separator or a commercially available platelet-concentrating separator, such as the Smart PreP system (Harvest Autologous Hemobiologics, Norwell, Massachusetts, USA), PCCS (3i Implant innovations, Palm Beach Gardens, Florida), or Curasan PRP kit (Curasan, Pharma Gmbh AG, Lindigstrab, Germany). These devices separate and concentrate platelets from small amounts of autologous blood, drawn preoperatively. To obtain PRP, two separate centrifugations, called spins, are required. The first spin, known as the separation spin, separates the red blood cells from the rest of the whole blood (white blood cells, platelets and plasma) (Figure 5-2). This is followed by a concentration spin, which separates the platelets, white blood cells, and a small number of residual red blood cells from the plasma (Figure 5-4).

This two-spin procedure results in PRP, which consists of a high concentration of platelets (4- to 5-fold increase over baseline platelet number in whole blood) and a native concentration of fibrinogen [65]. Prior to clinical application, the platelet concentrate is...
3.3. Cell adhesion molecules

Besides GFs, cell adhesion molecules (or extracellular-matrix molecules) have also been explored in the field of bone tissue engineering. It is known that certain peptides, (or extracellular-matrix molecules) such as laminin and fibronectin, available in serum, can enhance cellular attachment of various cell types, including bone cells, to synthetic surfaces (biomaterials) and hence, increase the bioactivity of these materials [66, 67]. This effect is mediated through integrin receptors on the cell surface. There are several types of integrins that are cell and tissue specific and bind to specific amino acid sequences [68, 69]. Osteoprogenitor cells carry integrin receptors, that bind to collagen and to certain peptides that contain a sequence of three amino acids (Arg-Gly-Asp), called RGD peptides. A selective activation of these receptors can preferably make bone forming cells become attached to the implant surface and thereby increase bone anchorage particularly in areas where the biological activity of the adjacent bone may be deficient [70]. Therefore, coating of biomaterials with RGDs offers an attractive strategy for controlling the cell-material interface and achieving a bioactive implant.

Other already clinically applied cell adhesion proteins, which are considered to enhance bone regeneration are ‘enamel matrix derivates’ (EMDs). EMDs are the major component of commercially available Emdogain® (Biora AB, Malmö, Sweden), which is approved for the treatment of periodontal defects. Emdogain® is a purified acidic extract of developing embryonic enamel derived from six-months-old piglets [71] (Figure 6).

Figure 6: Emdogain® : a) SEM; b) clinical formulation (Internet image, Straumann® Emdogain)

It is available in two formulations: a lyophilized form to be dissolved in an aqueous solution of PGA (propylene glycol alginate) prior to use and in a pre-mixed PGA-gel formulation. Both formulations contain 30 mg EMD protein/ml PGA gel, and are reported to have the same effects [72]. Due to the highly conserved structure and function of enamel matrix proteins among mammalian species and the exposure to these proteins in early childhood during tooth development, Emdogain®, although being of porcine origin, is considered “self-made” when encountered in the human body. Therefore, it can promote periodontal regeneration in humans without triggering allergic or immune responses or other undesirable reactions.

The major fraction of EMDs is composed of amelogenin, which is a cell-adhesion matrix-bound protein [73]. It is supposed to be active in epithelial-mesenchymal signalling and may also have direct signalling activities on cementoblasts and thus, on osteoblasts (73). Amelogenin, together with enamelin, sheathlin and 2 enzymes (MMP-20 and EMPS1) are present in Emdogain® and define its action, thereby the main biological effect is attributed to amelogenin. In general, EMDs are supposed to induce mesenchymal cells to mimic the events that occur during root development and thus to stimulate regeneration of periodontal tissues, i.e. cementum, periodontal ligament, and bone. The ability of EMDs to enhance bone formation in a nude mouse model were defined as osteopromotive [74] and were suggested to be due to the enhancement of the osteoinductive potential of the graft material by EMDs. However, it was also hypothesized that EMDs show osteopromotive characteristics because of the presence of bone growth stimulating factors [75], and a threshold concentration of EMDs is required to evoke an effect [76]. Although early immunoassay studies could not identify the presence of growth factors in EMDs [77], nominal levels of TGF-β1 [78] as well as BMP-2 and BMP-4 have been currently detected in an osteoinductive fraction of enamel extracts [79].

4. Objectives of the thesis

The present research is focused on the application of different biologically active additives for bone tissue regeneration. The final goal is to acquire more information about the efficacy of their bone regenerative properties and to develop a dedicated bone substitute that could be used in the clinic. The specific aims of this thesis are:

1. To summarize data available in the literature and provide evidence for clinical application of PRP in oral-maxillofacial bone regeneration;
2. To investigate the early effect of PRP on bone formation in combination with an osteoconductive material in a rat model;
3. To investigate the late effect of PRP on bone formation in combination with an osteoconductive material in a rat model;
4. To compare the effect of rat, goat and human PRP in combination with allograft and autograft in an immunodeficient rat model;
5. To examine the bone augmentation properties of TGF-β1 incorporated in an injectable PLGA/CaP composite in a rat model;
6. To evaluate the bone regenerative properties of EMD at osseous and non-osseous sites in a rat model.
References:


Chapter 2

The Effect of Platelet-Rich Plasma on Bone Regeneration in Dentistry: a Systematic Review

Adelina S. Plachokova, Dimitris Nikolidakis, Jan Mulder, John A. Jansen, Nico H. J. Creugers

Introduction

Platelet-rich plasma (PRP) is defined as an “autologous concentration of platelets in a small volume of plasma” (Marx 2004) and is considered to be a rich source of autologous growth factors. It was first introduced in 1998 by Marx and coworkers, in combination with autogenous bone grafts for reconstruction of mandibular defects (Marx et. al. 1998). Their study showed that the addition of PRP to bone grafts resulted in a faster radiographic maturation rate and higher bone density than bone grafts alone. Since then, the effect of PRP on bone regeneration has been extensively investigated. In the field of dentistry PRP has been used in different clinical procedures (i.e. sinus floor elevation, alveolar ridge augmentation, mandibular reconstruction, maxillary cleft repair, treatment of periodontal defects and treatment of extraction sockets), where it has been applied alone or in addition to autogenous bone, anorganic bone mineral and organic bone substitutes. Many papers reported positive effects of PRP on bone formation (Whitman et al. 1997, Kassolis et al. 2000, Camargo et al. 2002, Lekovic et al. 2003, Fennis et al. 2004, Nikolidakis et al. 2006). On the other hand, limited or no beneficial effect has also been shown (From et al. 2002, Monov et al. 2005, Raghoebhar et al. 2005, Thor et al. 2005, Plachokova et al. 2006).

The contribution of PRP to the bone healing process is thought to be based on the growth factors (GFs) stored in it. The following GFs are reported to be present in PRP: Platelet Derived Growth Factor (PDGF), Transforming Growth Factors-beta (TGF-ß), Vascular Endothelial Growth Factor (VEGF), Epithelial Growth Factor (EGF), Insulin Growth Factor-1 (IGF-1), basic Fibroblast Growth Factor (bFGF), as well as three blood proteins known to act as cell adhesion molecules for osteoconduction: (i.e. fibrin, fibronectin and vitronectin) (Tozum& Demiralp 2003, Sanchez et al. 2003). Therefore, PRP may influence bone formation through a variety of pathways.

The basic hypothesis of PRP addition to bone grafts is that high concentrations of platelets in a bony wound will increase the local concentration of secreted growth factors and subsequently enhance initial bone healing response. Later on, the direct influence of PRP will fade away and physiological mechanisms of bone repair will continue to work on an accelerated level (Jakse et al. 2003).

Currently, there is a lack of scientific evidence in the dental literature in favour of or against the clinical use of PRP for bone regeneration. Several reviews on the application of PRP in dentistry have been published (Schilephake 2002, Soffer et al. 2003, Tozum& Demiralp 2003, Sanchez et al. 2003, Marx 2004, Freymiller & Aghaloo 2004, Grageda 2004), but none of them followed a systematic approach. The aim of this systematic review is to determine and to structurally analyze the reported effects of PRP on bone regeneration in humans. For this purpose all clinical PRP applications in the field of dentistry will be considered.

Materials and Methods

A protocol was set-up prior to commencing the review, including agreements on search strategy, study inclusion criteria, data extraction, and statistical analysis.

The literature was searched for references published up to June 2006 in MEDLINE and Cochrane Central Register of Controlled Trial databases. Three search terms - “platelet-rich plasma”, “bone regeneration” and “dentistry”- were used together with their all known synonyms: “Bone regeneration” and “dentistry” were applied as MeSH terms, all the others as free-text terms. As a result, the following combinations were made: “platelet rich plasma” OR “platelet-rich plasma” OR “PRP” OR “autologous platelet gel” OR “plasma-rich growth factors” OR “autologous platelet concentrate” OR “platelet concentrates” AND “bone regeneration” OR “bone healing” AND “dentistry”. Abstracts of these references were read and controlled clinical trials meeting specific criteria were identified. Studies were included only if these (a) reported treatment outcomes (either by clinical, radiographic or/and histological measures) of PRP and controls on bone healing, (b) included at least 5 patients, and (c) had a follow-up of at least 3 months. Only papers written in English were considered. Papers not meeting all criteria were excluded. If no abstract was available in the databases, the abstract of the printed article was used. If title and abstract did not provide sufficient information to assess the eligibility of the paper, the full report was obtained as well. In addition, reference lists of previous published reviews were searched to detect additional studies. The search was carried out by two readers (A.P. & D.N.) independently. Disagreements were resolved by discussion.

In the following step the selected articles were printed and once again assessed by the two readers for the same inclusion criteria. For this purpose only Materials and Methods, and Results sections were used. Reference lists of papers included were checked by hand and cross-matched with the original list of references in order to detect additional studies that met the inclusion criteria. Relevant data (e.g. study design, patients’ characteristics, methods of PRP preparation, clinical outcomes, evaluation period) were extracted. Furthermore, study quality was assessed according to key quality components of controlled trials. Definitions for the quality assessment items were derived from two guidelines for systematic reviews (Cochrane Reviewers’ Handbook, 2001 and NHS Centre for Reviews and Dissemination, 2001, and Montenegro et al. 2002). Cohen’s Kappa-coefficient was used as a measure of agreement between the two readers in each step. Outcomes of the interventions in each study were compared. For this purpose Means and Standard Deviations (SD) or Standard Errors (SEM) drawn from each study were used to calculate Confidence Intervals (CI). The statistical analysis was performed by means of a commercially software program (SPSS for Windows version 12.0, SPSS Inc, Chicago, IL, USA).
Results

The MEDLINE and Cochrane search resulted in 108 hits. From assessing the titles and abstracts, ninety-one were excluded (inter-reader agreement $k = 0.89 \pm 0.06$) and finally, 17 articles were accepted for further analysis. One paper appeared to be unretrievable (Simon et al. 2004) resulting in sixteen articles remained to be read. Reading Materials and Methods and Results sections of the articles revealed 9 papers fulfilling the inclusion criteria (inter-reader agreement $k = 1$). Seven studies were excluded in this step: three of them were reviews and four had no control group. The hand search did not reveal additional studies to be included. All papers selected in the first selection step and the studies remaining after the second selection are presented in Table 1.

Table 1: Papers selected after first selection (in alphabetical order, $n=17$), papers remaining after second selection are presented in italics ($n=10$)

<table>
<thead>
<tr>
<th>References</th>
<th>Reasons for exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antilus 1999</td>
<td></td>
</tr>
<tr>
<td>Antilus 2005</td>
<td>Absence of control group, combined human &amp; animal study</td>
</tr>
<tr>
<td>Camargo et al. 2002</td>
<td>Absence of control group, improper study design to draw conclusions about PRP efficacy</td>
</tr>
<tr>
<td>Garg et al. 2000</td>
<td>Not a clinical trial (review)</td>
</tr>
<tr>
<td>Garg et al. 2001</td>
<td>Not a clinical trial (review)</td>
</tr>
<tr>
<td>Hanna et al. 2004</td>
<td></td>
</tr>
<tr>
<td>Kassolis &amp; Reynolds 2005</td>
<td>Improper control group</td>
</tr>
<tr>
<td>Marx et al. 1998</td>
<td></td>
</tr>
<tr>
<td>Okuda et al. 2005</td>
<td></td>
</tr>
<tr>
<td>Oyama et al. 2004</td>
<td></td>
</tr>
<tr>
<td>Raghoebert et al. 2005</td>
<td></td>
</tr>
<tr>
<td>Sammaritano et al. 2005</td>
<td></td>
</tr>
<tr>
<td>Simon et al. 2004</td>
<td></td>
</tr>
<tr>
<td>Steigmann &amp; Garg 2005</td>
<td>Absence of control group</td>
</tr>
<tr>
<td>Thor et al. 2005</td>
<td></td>
</tr>
<tr>
<td>Watt-Smith 2005</td>
<td>Not a clinical trial (review)</td>
</tr>
<tr>
<td>Wittfang et al. 2003</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 shows the extracted data according to different treatment modalities. A substantial heterogeneity in the studies existed with regards to patient populations, study designs, PRP preparation techniques, outcome measures and observation periods. The studies that reported similar outcome measures (i.e. clinical attachment level and percentage of new bone formation) were compared using the calculated Confidence Intervals (CI): Figures 1 and 2. PRP treatments of periodontal defects were significantly more effective compared to their controls in two studies (Okuda et al. 2005, Sammaritano et al. 2003). In the study of Hanna et al. the difference was very small and there was CIs overlap.

Table 2: Extracted data according to different treatment modalities

<table>
<thead>
<tr>
<th>Treatment modality</th>
<th>Reference</th>
<th>Number of patients</th>
<th>PRP preparation technique</th>
<th>Follow-up period (months)</th>
<th>Outcome measures</th>
<th>Outcome</th>
<th>Effect of PRP</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical procedure</td>
<td>Hanna et al. 2004</td>
<td>15</td>
<td>2-step</td>
<td>A &amp; CAL [mm]</td>
<td>6</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Okuda et al. 2004</td>
<td>10</td>
<td>3-step</td>
<td>A &amp; CAL [mm]</td>
<td>12</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Sammaritano et al. 2003</td>
<td>15</td>
<td>1-step</td>
<td>A &amp; CAL [mm]</td>
<td>12</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Regeneration</td>
<td>9</td>
<td>3-step</td>
<td>A &amp; CAL [mm]</td>
<td>3</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Milberg et al. 2003</td>
<td>15</td>
<td>2-step</td>
<td>A &amp; CAL [mm]</td>
<td>6</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Ota et al. 2005</td>
<td>8</td>
<td>1-step</td>
<td>A &amp; CAL [mm]</td>
<td>2</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Oota et al. 2005</td>
<td>8</td>
<td>1-step</td>
<td>A &amp; CAL [mm]</td>
<td>4</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Oota et al. 2005</td>
<td>8</td>
<td>1-step</td>
<td>A &amp; CAL [mm]</td>
<td>6</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Oota et al. 2005</td>
<td>8</td>
<td>1-step</td>
<td>A &amp; CAL [mm]</td>
<td>2</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Oota et al. 2005</td>
<td>8</td>
<td>1-step</td>
<td>A &amp; CAL [mm]</td>
<td>6</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Okuda et al. 2005</td>
<td>12</td>
<td>3-stages</td>
<td>A &amp; CAL [mm]</td>
<td>5</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Thor et al. 2005</td>
<td>12</td>
<td>3-stages</td>
<td>A &amp; CAL [mm]</td>
<td>12</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
<tr>
<td></td>
<td>Wittfang et al. 2003</td>
<td>20</td>
<td>3-step</td>
<td>A &amp; CAL [mm]</td>
<td>6</td>
<td>Percentage CAL &amp; A</td>
<td>Positive</td>
<td>Bone grafting, Acrylic, Calcium Sulfate</td>
</tr>
</tbody>
</table>

Table 2: Comparison of the Mean values and 95% Confidence Intervals for test and control groups of the selected studies performed for treatment of periodontal defects. The results of ΔCAL in mm are presented. The means of the controls were set on 0. Plus (+) values show favorable effect of PRP.
With respect to the use of PRP in sinus elevation, one study (Raghoebar et al. 2005) reported no difference compared to the control. The study of Wiltfang et al. 2003 reported a positive effect of 9%, however there was a substantial overlap in the CIs. Overall, no significant difference in the treatment outcome was observed between sinus elevation procedures with and without PRP.

Table 3 presents the outcomes of the study quality assessment (inter-reader agreement \( k = 0.90 \pm 0.04 \)). Overall, the majority of them do not meet current recommendations on study quality related to sample size calculation, randomization methods, allocation concealment, examiner blinding, validity of statistical methods and validity of outcomes and estimation.

**Table 3:** Quality assessment of selected studies (n=9)

<table>
<thead>
<tr>
<th>Quality assessment items*</th>
<th>Yes/Adequate n</th>
<th>No/Inadequate n</th>
<th>Unclear n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size calculation described</td>
<td>8</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Described as controlled</td>
<td>6</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Described as randomized</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Randomization methods</td>
<td>1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Allocation concealment method</td>
<td>6</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Examiner blinding</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients follow-up</td>
<td>5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Validity of statistical methods</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

Discussion

This systematic review aimed to assess the available scientific evidence for applying PRP for bone regeneration in all fields of dentistry. The inter-reader agreements for inclusion of papers and quality assessment are unusually high (i.e. first step \( k = 0.89 \pm 0.06 \), second step \( k = 1 \), quality assessment \( k = 0.90 \pm 0.04 \)) most probably because both readers are well acquainted with the subject of PRP (Plachokova et al. 2006, Nikolidakis et al. 2006). Unfortunately, no statistical analysis of the data was possible due to the heterogeneity of the studies. However, the present study is considered a qualitative systematic review since rigorous methods for outcome assessment were applied (Sutherland 2004). At the same time a risk of publication bias has to be mentioned, since English language was used as a limit by the literature search. Moreover, unpublished data, as well as commercial interests in the studies were not explored.

An attempt was made to compare results from the studies that used similar outcome measures by adding Confidence Intervals to the data presented in the original papers (Figure 1 and Figure 2). On one hand, it was not allowed to pool these data because of different follow-up periods. On the other hand, the similar and objective presentation of the measured outcomes from the different studies enabled a relative comparison. Differences in treatment effects for periodontal defects in terms of clinical attachment level (CAL) were significant, the mean differences ranging from 0.8 to 3.2 mm, however, the differences in the CI in two out of three studies appeared to be small (Hanna et al. and Okuda et al). An explanation for the superior results of Sammartino et al. 2005 (above the rest of the studies) could be the shorter evaluation period used (3 months versus 6 and 12 months). PRP is supposed to exert its effect on the early stages of bone formation, accelerating the bone healing process, therefore it could be speculated that at later time points this effect diminishes or does not exist any more. Besides that, PRP in the study of Sammartino was used to treat periodontal defects caused by the complications of mesioangular impacted mandibular third molar, whereas in the studies of Hanna et al. and Okuda et al. PRP was applied in defects caused by periodontal disease. In addition, the age effect of the patients enrolled in the studies, as a factor influencing the treatment outcomes, could not be excluded. In the study of Sammartino patients’ ages ranged between 21 and 26, whereas in Hanna et al. between 35 and 75 years. The mean age reported by Okuda et al. was 55.5 ± 8.2.

Another explanation for the overall positive effects reported by the periodontal studies could be the methods of assessment used (CAL and PD measurements). It is known that besides to bone formation, gain in CAL is due to formation of a connective tissue fibers and long junctional epithelium. Since it is claimed that PRP has also stimulatory
effect on fibroblasts (Marx et al. 1998), it could be the PRP enhanced connective tissue proliferation that caused the positive changes in the outcome measures. Clinically it is not possible to discern how much of the gain in CAL is due to bone formation and how much to connective tissue ingrowth. Only histological evaluation can provide this information. Unfortunately, re-entry procedures to obtain histological samples are not a routine practice, especially when periodontal regeneration is aimed/perceived. In this regard, Zybutz et al. 2000 compared clinical (PD and CAL) and radiologic measurements of intrabony defects to direct bone measurements during surgical procedure, and concluded that probing attachment level measurements (CAL) are accurate methods of assessing bone level and can be reliably used to assess bone level changes after treatment (Zybutz et al. 2000). This finding may validate the conclusions of the above-mentioned studies, which results are based only on the changes of CAL and PD over time. However, it has to be emphasized that histological evaluations are needed to corroborate the bone regenerative effect of PRP.

In view of the sinus elevation procedures, the reported effects of PRP (compared to their controls) were less than 10%. Moreover, there was a wide variety in outcome, which is demonstrated by the large Confidence Intervals (> 10%). Consequently, the evidence for a beneficial effect of PRP in sinus elevation is considered to be weak. This corroborates the conclusions of the review of Boyapati and Wang (Boyapati & Wang 2006). Based on animal and human studies they concluded that the additional use of PRP in sinus augmentation could not be recommended.

Overall, the majority of the reviewed studies reported positive effect of PRP for bone regeneration. However, the quality assessment of these studies, judged by their reports, revealed that most of them do not meet current recommendations on study quality. Study designs, randomization methods, concealments of the allocation sequence and blinding of examiners could not be judged adequately. In some studies the validity of outcomes and estimation was found unclear or even inadequate. If these methods are not implied or properly executed in the actual study conduct, outcomes of these trials could be biased. Inadequate or unclear methods were found to exaggerate the treatment effect compared with adequate methods (Juni et al. 2001; Colditz et al. 1989). In view of the above mentioned, the data provided by poor quality studies as judged by their publications, can not be used as evidence. To increase evidence, better reporting of clinical trials on PRP is required, and the CONSORT statement (Moher et al. 2001b) is suggested as a helpful guide for improving study quality.

In this review a substantial heterogeneity among the studies was observed. One of the reasons is the difference in protocols as used for PRP production. Some studies used commercially available platelet-concentrating separators (FDA approved and/or European recognized) for PRP production, others used general-purpose cell separators. It has been claimed that the latter can be acceptable only when a standardized, two step spin procedure is followed. Single-spin machines have been reported to be incapable of separating and concentrating platelets to a therapeutic level (Marx & Garg 2005). Some of the included studies using the non-FDA-approved devices failed to provide a detailed description of the protocol for PRP production. In general, preferably this description should include information about the cell-separator used (name company, country), centrifugation steps (rotations per minute, g/minutes), amount of blood collected preoperatively, baseline number of platelets in the collected blood, amount of PRP obtained, number of platelets in the obtained PRP (increase above baseline). In addition, the use of coagulation promoters (doses, mixing ratio to PRP) is considered indispensable information. In the current systematic review papers were not excluded for this reason since the omissions in the descriptions were only limited. However, the inclusion of above-mentioned parameters has to be recommended.

In summary, evidence was found for the use of PRP in periodontal defects. The evidence available in the dental literature for beneficial effects in sinus elevation is weak. No conclusions can be drawn about PRP for other applications in dentistry.

Acknowledgements

We would like to thank Vincent Cuijpers for his help with the figures.
References:


Chapter 2


Chapter 3

The Early Effect of Platelet-Rich Plasma on Bone Healing in Combination with an Osteoconductive Material in Rat Cranial Defects

Adelina S. Plachokova, Juliette van den Dolder, Paul J Stoelinga, John A. Jansen.

Introduction

In order to create a bone graft, which is comparable or superior to natural bone, allografts are combined with osteoinductive substances, such as recombinant growth factors (GFs) (Jansen et al. 2005). A disadvantage of the recombinant GFs is that they are expensive. In view of this, platelet-rich plasma (PRP), as a rich source of autologous growth factors, is considered to be an alternative approach (Marx et al. 1998). The basic hypothesis of the addition of PRP to bone grafts is that a high concentration of platelets in a bone defect will increase the local concentration of secreted GFs and subsequently enhance initial bone regeneration (Marx et al. 1998). After a few days, the direct influence of the administered PRP will fade away and physiological mechanisms of bone repair continue to work on an accelerated level (Jakse et al. 2003). The following GFs are reported to be present in PRP: Platelet Derived Growth Factor in its three isoforms (PDGFαα, ββ, ααββ), Transforming Growth Factors-beta (TGF-β1 and TGF-β2), Vascular Endothelial Growth Factor (VEGF) and Epithelial Growth Factor (EGF), as well as three blood proteins known to act as cell adhesion molecules for osteoconduction: i.e. fibrin, fibronectin and vitronectin (Marx et al. 1998; Marx 2004). According to some authors PRP is also a storage vehicle for basic Fibroblast Growth Factor (bFGF), Insulin Growth Factor-1 (IGF-1), Platelet Factor-4 (PF-4), and interleukin-1 (Aghaloo et al. 2002; Tozum & Demiralp 2003; Sanchez et al. 2003). All this implies that PRP might influence bone formation through a variety of pathways.

Calcium phosphate ceramics, e.g. hydroxyapatite (HA) and tricalcium phosphate (TCP) are frequently used for bone reconstruction, because of their close resemblance to the bone mineral phase (Dalcusi et al. 2003; El-Ghannan 2005). Both HA and TCP are biocompatible and osteoconductive, but at the same time have their disadvantages. Previous studies revealed that HA particles are a good bone filler for new bone formation, but they are poorly resorbable. On the other hand, TCP tends to resorb unpredictably in biologic fluids and in a variety of solvents (Dalcusi et al. 2003). The fabrication of biphasic calcium phosphate ceramic (BCP) made it possible to control the resorbability of the material and at the same time to maintain its osteoconductive property. In a previous case report it was shown that a ratio of 60% HA to 40% β-TCP provides a bone conductive surface (Ellinger et al. 1986). This was confirmed in a previous study of our group using dense HA/β-TCP (60%/40%) particles, with a size of 300 - 500 μm to fill cranial defects in rats (Plachokova et al. 2006). At the same time we hypothesized in our previous study that such particles could be a good delivery vehicle for the growth factors present in PRP. Unfortunately, after an implantation period of 4 weeks no effect at all of PRP on bone formation was demonstrated, irrespective whether PRP was applied in form of a liquid or gel (Plachokova et al. 2006). However, due to the fact that the mitogenic properties of the cytokines present in PRP act during the first 1-2 days and the life span of the platelet itself is less than 5 days, a substantial impact of PRP on bone regeneration might only be observed during the early stages of bone healing (Malaval et al. 1994; Kessler et al. 2000). Moreover, the rat cranial defects were almost completely closed after 4 weeks, which could have prevented PRP to demonstrate its positive influence. Therefore, we decided to investigate the effect of PRP on bone regeneration for an implantation time of 1 and 2 weeks.

In summary, in this study we hypothesize that PRP exerts its beneficial effect on bone regeneration within the first and second week after application in a bone defect combined with an osteoconductive material.

Materials and Methods

Bone fillers

Dense HA/β-TCP (60%/40%) particles with a size of 300-500 μm (Camceram, CAM implants, Leiden BV, the Netherlands) were used as synthetic bone substitute. Calvarial defects were filled with: (1) particles alone (n = 8), and (2) particles combined with PRP gel (n = 8), with a mixing ratio of 1 gram HA/β-TCP particles to 150 μl PRP. Some defects were also left unfilled, which served as controls (n = 8). HA/β-TCP particles mixed with PRP were always applied directly after preparation.

PRP preparation

The whole blood of four male syngenetic Fisher 344 rats was drawn preoperatively via cardiac puncture into tubes containing 3.8% sodium citrate. Platelet-rich plasma (PRP) was obtained from this anticoagulated blood after centrifugation at 800 rpm for 15 minutes at 25°C. The platelets present in the whole blood as well as present in PRP were counted automatically using a hematology analyzer (Advia 120, Bayer B.V., Mijdrecht, the Netherlands). The concentration of platelets obtained in the PRP was 3 times higher than the baseline. The PRP was used as a PRP gel. Therefore, 150 μl of the platelet concentrate was activated at the time of surgery with 25 μl of 10% calcium chloride solution and 25 μl of 300 IU of bovine thrombin (300 IU) (FibriniquitTM Thrombin, BioMerieux Inc., Durham, NC, USA).

Surgical procedure

Forty-five, 11 - 12 weeks old syngenetic male Fisher 344 rats, each weighing between 220 and 260 g, were used in this study. National guidelines for the care and use of laboratory animals were observed. Anesthesia was induced with 4% Isoflurane and maintained with 2% Isoflurane and...
0.4% N₂O, 0.4% O₂ by non-rebreather mask and monitored to ensure that an appropriate level of anesthesia was achieved and maintained for surgery. The animals were premedicated by an intramuscular injection of Fentanyl (3 ml/kg) (Hamelink Pharmaceuticals GmbH, Germany) to reduce the operative pain, and a subcutaneous injection of Buprenorphine (150 μg/kg) (Temgesic®, Reckitt Benckiser Healthcare Limited, UK) was applied to reduce the postoperative pain. Before surgery, the dorsal part of the rat cranium was shaved and swabbed by iodine. A median sagittal incision extending from the nasofrontal area to the occipital protuberance was made and soft tissues were sharply dissected to visualize the cranial periosteum. The periosteum was then undermined and reflected, exposing the parietal bones. A hollow trephine bur with an outer diameter of 6.2 mm in a dental hand piece was used to create a full-thickness defect into the dorsal part of the parietal bone. Based on literature, we supposed that a 6.2 mm cranial defect would be a critical-sized defect in rats for one or two weeks of implantation.

Bosch et al. (1998) and Verna et al. (2002) described 5 mm defects as critical-sized in testing materials for bone regeneration during implantation periods of 6 and 12 months. During the surgical procedure, the mid-sagittal suture was avoided in order to reduce the risk of damaging the superior sagittal sinus and to minimize the morbidity of the animals (Bosh et al. 1998; Verna et al. 2002). Care was also taken not to damage the dura mater or to puncture the superior sagittal sinus. Dense HA/β-TCP particles alone or in combination PRP gel were placed in a 6.2 mm cranial defect. Empty defects were used as control. For the implantation time of 1 week, seven defects were made in each group: empty defect (n = 7), HA/β-TCP alone (n = 7) and HA/β-TCP + PRP (n = 7). For the implantation time of 2 weeks, eight defects were made in each group: empty defect (n = 8), HA/β-TCP alone (n = 8) and HA/β-TCP + PRP (n = 8). After insertion of the various materials, the periosteum and overlying skin were closed in separate layers with 5-0 and 4-0 Vicryl resorbable sutures.

**Histological preparation**

The animals were euthanized 1 and 2 weeks after surgery using an overdose of carbon dioxide. The skin was dissected and the defect sites were removed along with surrounding bone and soft tissues, and fixed in 10% neutral formalin for one week. Part of rounding bone and soft tissues, and fixed in 10% neutral formalin for one week. First, specimens were used for μCT, thereafter specimens were used for histology. The two remaining specimens were left undecalcified (n = 2, both for 1st and 2nd week) and were embedded in polymethylmethacrylate (PMMA). Before the embedding procedure, an incision was made exactly through the middle of the bone defects to secure that the micrometre sections were made in the area of interest. Sagittal paraffin sections, 5 μm in thickness, were stained with haematoxylin-eosin, and trichrome staining (Masson modification Goldner). Methylene blue and basic fuchsin were used as a staining for the PMMA samples, which were prepared using a modified diamond-blade sawing micromotive technique and were 10 μm thick. Light microscopical analysis consisted of descriptive histology and was made using an optical microscope (Leica BV, Rijswijk, the Netherlands).

A scoring system was used to quantify defect closure. The scoring scale for the defect closure ranged from 0 to 4, where 0 represented a completely empty defect (0% closure), 1 corresponded to 25% defect closure, 2 to 50% defect closure, 3 to 75% defect closure and 4 to 100% defect closure.

**μCT scan analysis**

Before histological preparation, computerised X-ray microscopy and tomography of the samples (n = 6) was performed by μCT scanner with a very high spatial resolution (5 μm corresponding to near 1 x 10⁷ cubic mm voxel size) (Sky Scan 1072, SkyScan NV, Aartselaar, Belgium). For each sample, a total of 336 micro-tomographic slices were gained using a slice increment of 25 μm in order to scan the whole defect (6.2 mm) and the surrounding bone. From the μCT scan datasets, 3D models were built for visualization and morphometric analysis. For the morphometric analysis, a subregion of the originally measured data was selected. This selected region of interest (ROI) included the whole defect area with the newly formed bone inside. By auto-interpolation of layers, ROI became volume of interest (VOI), which is the essential basis for all quantitative analysis. VOI was determined to be 15 mm³. It was calculated in advance using the formula V = π r² h, where “r” is half of the diameter size of the original defect and “h” represents the thickness of the skull measured interactively or by a caliper. Images from only the axial projection of the skull were taken, which resulted in 207 layers covering the whole defect. It turned out that VOI of 15 mm³ with a pixel size of 21.88 μm corresponded to 207 layers. Within the VOI, the volume of the particles and the volume of the newly formed bone were calculated as a percentage or as volume of the newly formed bone, and particles, respectively.

It was possible to identify accurately the newly formed bone from the HA/β-TCP particles when the raw data reconstructed cross-sections were turned into images. Each image used 256 gray scales. Based on histogram calculations, the bone was defined to be in the 64 - 225 grey value range, whereas the particles were regarded to lie between 0 and 63.
Statistical analysis
For the statistical analysis GraphPad InStat® (GraphPad Software, Inc.) was used. One-way Analysis of Variance (ANOVA) was performed on the raw data obtained from μCT analyses. ANOVA assumes that the data are sampled from populations with identical standard deviations. This assumption was tested using the method of Bartlett. Further, ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption was tested using the method Kolmogorov and Smirnov. In addition, Tukey-Kramer Multiple Comparisons Test was done.

Results
All animals had an uneventful recovery. Their weight remained stable and increased during the implantation period, whereas no infection occurred during that time.

Descriptive histology
One week results
Light microscopic examination of the decalcified sections showed that bone formation had already started in the 1st week for all experimental groups. Newly formed bone could be observed at the defect borders and at the side of the dura (Figure 1).

Figure 1: Start of bone formation during the 1st week mainly at the side of the dura. Undecalciﬁed samples embed- ded in PMMA and stained with Methylene Blue-Basic Fuchsin, 2.5 x magnification. A) empty defect, B) defect with particles, C) defect with particles and PRP. * newly formed bone; # old skull bone; p particles.

Two weeks results
After two weeks, complete bridging of the bone defect was observed for most of the empty defects, as well as for the defects that contained only HA/β-TCP particles. The HA/β-TCP filled defects seemed to support more bone formation in comparison to the PRP/HA/β-TCP filled defects. On the other hand, the layer of newly formed bone in the empty defects was thinner than the layer of newly formed bone in both particle groups (Figure 2).

Figure 2: Bone formation after 2 weeks. The layer of newly formed bone in the empty defects is thinner than the layer of newly formed bone in both particle groups. Decalcified paraffin samples stained with hematoxylin-eosin (H.E.), 2.5 x magnification. A) empty defect, B) defect with particles, C) defect with particles and PRP. * newly formed bone; # old bone; p particles.
Examination of the PRP and non-PRP samples revealed that in both groups some of the HA/β-TCP particles were almost completely surrounded by bone without an intervening fibrous tissue layer. Evidently, newly formed bone was guided over the outer surfaces of the particles (Figure 3).

The trichrome staining showed in general more mature bone (stained in red) in the second week compared to the first week (stained in green) (Figure 4).

No difference in vascularisation was observed subjectively in the number of the blood vessels among all groups. However, neovascularisation was more evident after 2 weeks.

The histological findings of the non-decalcified samples were again comparable with the decalcified sections.

**μCT analysis**

Bone formation in the skull defects (n = 6, randomly selected) was also imaged with μCT. μCT images confirmed the uniform distribution of the particles throughout the defects for both PRP and non-PRP groups. Only occasionally, some particles were observed outside the defect.

Although the tomographic X-ray images suggested that no bone formation had occurred neither in the empty nor in the grafted defects, the 3D reconstructed representations demonstrated a completely different result. In these reconstructions, bone formation could already be detected after the 1st week of implantation in all experimental groups. The 3D models of the filled defects showed penetration of the newly formed bone between the particles already after one week. At the second week, the bone ingrowth from the side of the dura and the defect borders was especially prominent. Also, in the empty defects start of new bone formation was observed after one week, whereas some of the defects were almost completely closed after 2 weeks (Figure 5B, C).

The data from the morphometric analysis are presented in Figure 6 and show that the amount of newly formed bone after 1 week in the ROI, expressed as relative volume, ranged from 3.57% (empty defects) to 14.86% (HA/β-TCP group). This difference was statistically significant (p < 0.01). On the other hand, no significant difference existed between the PRP and non-PRP HA/β-TCP particulate groups.
At the second week, the volume of the newly formed bone was increased to 14.5% for the empty defects, 19.9% for the HA/β-TCP and 17.2% for the HA/β-TCP + PRP. Statistical analysis revealed that these differences were not statistically significant (p = 0.1745).

A comparison of the results from both implantation times showed that there was a significant difference in the bone formation rate for the group with empty defects between the 1st and 2nd week. However, no statistically significant difference in the amount of the newly formed bone was observed between both particulate groups at 1 and 2 weeks of implantation.

Discussion

Platelets and platelet-derived growth factors are known to be effective during the early stage of bone graft healing, because the life span of a platelet in a wound and the period of direct influence of its GFs are less than 5 days (Choi et al. 2003). Therefore, a pronounced effect of PRP is supposed to occur especially during the early stages of bone regeneration. However, in our study, after 1 and 2 weeks of implantation of HA/β-TCP particles mixed with PRP in rat skull defects, no significant difference in bone formation between the PRP and non-PRP groups was observed, neither histologically nor by μCT evaluation. Evidently, no positive effect of PRP on bone formation could be confirmed. This corroborates the study of Butterfield et al. (2005), who performed maxillary sinus augmentation in rabbits with autogenous bone graft and PRP. After 2, 4 and 8 weeks of implantation Butterfield et al. also failed to prove a direct stimulatory effect of PRP on bone healing. On the other hand, they found a statistically significant difference in total bone area and bone formation rate between 2 and 4 weeks in the PRP group. This founding is in contrast to the hypothesis that most of the effect of PRP will occur during the very early healing period. These results agree also with the summary of the μCT analysis from our previous (Plachokova et al. 2006) and current study.

On the other hand, our findings contradict those of Schlegel et al. (2004), who reported a significant accelerating effect of PRP on early bone regeneration (2 weeks) in combination with autogenous bone applied in the forehead area of pigs. At the 2nd week, they showed microradiographically a maximum rate of 63% mineralization in the PRP groups and this peak level occurred 2 weeks earlier than in the group using autogenous bone alone. In addition, more new blood vessels were observed histologically when PRP was applied. Several explanations can be given for this discrepancy in observations. Firstly, the technique to prepare PRP and the concentration of the gained thrombocytes can differ. Schlegel et al. (2004) managed to achieve a 4.1- and 6.5-fold increase of pig platelets above the baseline. The rat PRP that we produced had 3-fold increase. Nevertheless, despite the smaller number of platelets in the rat PRP, a substantial number of GFs have been proven to be present there (unpublished data). Secondly, differences between the used animals (age, breed) can play a role. Thirdly, better results with PRP are reported when it is combined with autogenous grafts and not with allogenic materials (Wiltfang et al. 2004).

Considering the results of the orthotopic application of dense HA/β-TCP particles, our current findings are in keeping with our previous study (Plachokova et al. 2006). It was confirmed again that dense HA/β-TCP particles did not cause any inflammatory reaction. It was also evident again that a 6 mm cranial defect in Fisher rats is not a critical-sized defect (CSD), since at two weeks most of the empty defects were already closed with bone tissue. Although, some other reports claim that defects with a diameter of 4 - 5 mm have to be considered as CSDs (Mulliken & Glowacki 1980; Bosch et al. 1998), our data demonstrated that a full thickness defect with a diameter of 6.2 mm in rat calvaria shows a tendency for almost complete closure within 2 weeks (Figure 5C). An explanation for this inconsistency in defect closure versus defect diameter could be the evaluation technique as used in various studies. For example, Duporieux et al. (1999, 2001) created also 6 mm sized calvarial defects in Wistar rats with the same weight as in our study (between 250 and 300 g). They suggest that empty control defects are still open after healing periods of 6 - 9 weeks. However, their conclusion is based on a radiographic analysis. In the current study, but also in an earlier publication (Hedberg et al. 2005), we observed that an incongruity exists between the 2D X-ray image and a 3D μCT reconstruction. New bone formation was observed after the 1st week in a 3D model, while it was not possible to detect bone formation on the tomograms. In view of this, it has to be emphasized that only 3D models based on computerized tomograms, as made of the complete defect site, can visualize the final spatial distribution of the newly formed bone and the direction of the bone formation over the total defect area.

Finally, in our present study the options of different μCT software programs for morphometric analysis were explored more profoundly. Basically, the same approach was applied as in our previous study (Plachokova et al. 2006). The difference was that this time images were taken only from the axial projection of the skull and not from the transversal, which resulted in 207 layers covering the whole defect. It appeared that the same VOI of 15 mm³ with pixel size of 21.98 μm corresponded to 207 layers. Apart from the larger number of slices that we obtained, 207 in comparison to 20, it was easier to follow the ROI in an axial aspect. Another advantage of this approach was that ROI was drawn by hand (i.e. the polygonal freehand drawn ROI) independently for each layer,
which enabled a more precise position of the borders of the ROI. Despite the introduced changes, the results that we obtained from the current morphometrical analysis were consistent with the results from our previous study. All the data were put together in Figure 6 to follow the trend of bone regeneration, which appeared to be linear. All this implies that irrespective of the used algorithm, currently the μCT analysis remains a very reliable source of information in the field of bone research.

Conclusion

Based on our results, we conclude that a 6.2 mm cranial defect is not a critical-sized defect in rats. Further, to our knowledge this is one of the few studies that investigated the early effect of PRP on bone regeneration. However, no effect at all of rat PRP on healing of cranial bone defects in combination with synthetic bone substitute was observed. Further, dense HA/β-TCP (ratio 60%/40%) particles with a size of 300-500 μm were found to have a beneficial effect on bone formation already after 1 and 2 weeks of implantation in rat cranial defects.

Acknowledgement

We would like to thank Natasja van Dijk for her help with the histology and Cees Verhagen, Sanquin Zuid-Oost, Nijmegen for the PRP protocol. Further, we would like to thank Dolf Lubbers, Department of Hematology, UMC Nijmegen for counting the platelets with a hematology analyzer (Advia 120, Bayer B.V., Mijdrecht, The Netherlands).

References


Introduction

In an attempt to create a bone graft, which is comparable or superior to bone, allografts are combined with osteoinductive substances, such as growth factors (GFs). Several recombinant GFs have been produced and numerous studies have shown their beneficial impact on bone healing. Besides the demonstrated positive effects of human recombinant GFs in bone regeneration, there are also some disadvantages to be overcome, such as short shelf-life, inefficient delivery to target cells, high price, and dose-dependent effect.

Platelet-Rich Plasma (PRP), as a rich source of autologous growth factors, can be regarded as an alternative approach. It was first introduced in 1998 by Marx et al. in combination with autogenous bone grafts for reconstruction of mandibular continuity defects. Their study showed that addition of PRP to bone grafts resulted both in a faster radiographic maturation rate (1.62 to 2.16 times) and higher bone density than the control group (Marx et al. 1998). Since then, the effect of PRP on bone regeneration has been extensively investigated, while also different techniques to prepare PRP have been developed. Clinical studies and case reports on sinus floor elevation, alveolar ridge augmentation, mandibular reconstruction, maxillary cleft repair and periodontal regenerative procedures have already described the beneficial effect of PRP in addition to autogenous bone, anorganic bone mineral, organic bone substitutes or when used alone (Whitman et al. 1997; Obbario et al. 2000; Kassolis et al. 2000; Camargo et al. 2002; Fennis et al. 2002, 2004; Lekovic et al. 2003; Rodriguez et al. 2003; Wiltfang et al. 2002). On the other hand, some experiments showed only a limited or no beneficial effect of PRP on bone formation. Schmitz & Hollinger (2001) even expressed their concern about the insufficient biological understanding of the clinical therapy surrounding PRP. They pointed out that the exact effects of PRP on bone formation are still unknown and suggested further research.

The basic hypothesis of the addition of PRP to bone grafts is that a high concentration of platelets in a bony wound will increase the local concentration of secreted GFs and subsequently enhance initial bone regeneration. After a few days, the direct influence of the administered PRP will fade away and physiological mechanisms of bone repair continue to work on an accelerated level (Jakse et al. 2003). The following GFs are reported to be present in PRP: Platelet Derived Growth Factor in its three isoforms (PDGFαα, ββ, αβ), Transforming Growth Factors-beta (TGF-β1 and TGF-β2), Vascular Endothelial Growth Factor (VEGF) and Epithelial Growth Factor (EGF), as well as three blood proteins known to act as cell adhesion molecules for osteoconduction: fibrin, fibronectin and vitronectin (Marx et al. 1998; Marx 2004). According to some authors PRP is also a storage vehicle for basic Fibroblast Growth Factor (bFGF), Insulin Growth Factor-1 (IGF-1), Platelet Factor-4 (PF-4), and interleukine-1 (Aghaloo et al. 2002; Tozum & Demiralp 2003; Sanchez et al. 2003). All this implies that PRP might influence bone formation through a variety of pathways.

In our study, we hypothesized that PRP might possess osteoinductive properties and thus applied in addition to HA/β-TCP as a scaffold material may enhance bone formation. It was decided to compare the effect of PRP in form of a liquid and PRP in form of a gel, to see if the addition of the clot formation accelerators, thrombin and calcium chloride, influences the bone formation and adds to the initial stability of the implant. This is one of the first studies that investigates whether there is a difference in the activity of PRP either in a gel or a liquid form, while combined with biphasic ceramic particles. Besides histology, specimens were evaluated using high resolution X-ray computed tomography (μCT). Decision was taken to combine PRP with biphasic ceramic particles composed of 40% beta-tricalcium phosphate (β-TCP) and 60% hydroxyapatite (HA) (Camceram, CAM Implants, Leiden, the Netherlands), because this material is well studied and is already in clinical use as a bone graft substitute. HA/β-TCP is osteoconductive and slowly degradable.

Materials and Methods

Bone fillers
Dense HA/β-TCP (60%/40%) particles were used as synthetic bone substitute with a size of 300-500 μm (Camceram CAM Implants, Leiden, the Netherlands). For the animal group, where the defects were filled with particles alone, 1 gram of the particles was mixed immediately before implantation with 100 μl blood. When particles were inserted together with PRP liquid, the mixing ratio was 1 gram HA/β-TCP particles to 150 μl PRP. The same ratio was used when HA/β-TCP particles were combined with PRP gel. All the mixtures were prepared in the immediate preoperative time and applied right away.

PRP preparation
The whole blood of four male Fisher 344 rats was drawn preoperatively via cardiac puncture into tubes containing 3.8% sodium citrate. PRP was obtained from this anti-coagulated blood after centrifugation at 800 rpm for 15 minutes at 25° C. Three mL of blood resulted in 150 μl of PRP. The platelets present in the whole blood and present in PRP were counted automatically using a hematology analyzer (Advia 120, Bayer BV, Mijdrecht, the Netherlands). The concentration of platelets achieved in the PRP was 3 times higher than the baseline. The PRP was used as prepared or to obtain PRP gel.
Therefore, the platelet concentrate was activated at the time of surgery with a 10% calcium chloride solution and 300 IU of bovine thrombin. One hundred and fifty microliters of PRP was mixed with 25 μl Thrombin (300 IU) (Fibriquik™ Thrombin, BioMerieux Inc., Durham, NC, USA) together with 25 μl 10% CaCl₂.

**Surgical procedure**

Thirty-eight syngeneic male Fisher 344 rats, each weighing between 220 and 260 g, were used in this study. National guidelines for the care and use of laboratory animals were observed.

Dense HA/ß-TCP particles alone or in combination with PRP liquid or PRP gel (n = 10) were placed in a 6.2 mm cranial defect. Eight defects (n = 8) were left empty as control. In this way, four groups were created, i.e. empty defects, defects with HA/ß-TCP particles, HA/ß-TCP particles combined with PRP and HA/ß-TCP combined with PRP gel.

Anesthesia was induced with 4% Isoflurane and maintained with 2% Isoflurane and 0.4% N₂O, 0.4% O₂ by non-rebreather mask and monitored to ensure that an appropriate level of anesthesia was achieved and maintained for surgery. To reduce the post-operative pain the animals were premedicated by an intramuscular injection of Fentanyl (3 ml/kg) and subcutaneous injection of Buprenorphine (150 μg/kg). Before surgery, the dorsal part of the rat cranium was shaved and swabbed by iodine. A median sagittal incision extending from the nasofrontal area to the occipital protuberance was made and soft tissues were sharp dissected to visualize the cranial periosteum. The periosteum was then undermined and reflected, exposing the parietal bones. A hollow trephine bur with an outer diameter of 6.2 mm in a dental hand piece was used to create a full-thickness defect (Bosch et al. 1998) into the dorsal part of the parietal bone (Fig. 1).

**Histological preparation**

The animals were euthanized 4 weeks after surgery using an overdose of carbon dioxide. The skin was dissected and the defect sites were removed along with surrounding bone and soft tissues, and fixed in 10% neutral formalin for one week. After that, the specimens were decalcified in Formical-2000 (Immunodiagnostika & Biotech GmbH, Berlin, Germany) for one week and then embedded in Paraplast paraffin (Klinipath B.V., Duiven, the Netherlands). Before the embedding procedure, an incision was made exactly through the middle of the bone defects to secure that the microtome sections were made in the area of interest.

Sagittal sections of about 5 μm in thickness were prepared, stained with haematoxylin-eosin and observed under an optical microscope (Leica BV, Rijswijk, the Netherlands). Light microscopical analysis consisted of descriptive histology and histomorphometrical analysis.

**Histomorphometry**

For histomorphometry a scoring system was used to quantify defect closure and bone bridging at the defect border. The scoring scale for the defect closure ranged from 0 to 4, where 0 represented a completely empty defect (0% closure), 1 corresponded to 25% defect closure, 2 to 50% defect closure, 3 to 75% defect closure and 4 to 100% defect closure.

Bridging at the defect border was defined as the occurrence of bone formation between the defect border and the HA/ß-TCP particles in the defect. The grading scale for the bridging at the defect border ranged from 0 to 2, where 0 stands for no bridging, 1 for bridging at one defect border and 2 for bridging at two defect borders.

Further, image analysis was performed on three digitalized sections per specimen to quantify the bone area within the defect, which is considered to be the newly formed bone. For this purpose, a Leica® Qwin Pro-image analysis system (Leica BV, Rijswijk, the Netherlands) was used and sections were digitized at low magnification. The newly formed bone, as defined by its woven structure and location, was marked in an interactive manner, and the computer calculated its surface area (BA) in mm² (Fig. 2a). Another parameter to determine was the region of interest (ROI) in mm², defined as the surface of the area between the defects borders that had to be filled with the newly formed bone (Fig. 2b). For the calculation of ROI, the thickness of the skulls was taken into account, as they were measured in advance using a caliper. From these two data,
the bone fill percentage (%) i.e. the index of the bone surface area (BA) divided by ROI was calculated. Presented results are based on the average of these measurements.

Figure 2: Histomorphometric measurements:
A) newly formed bone marked in an interactive manner and defined as bone surface area (BA)
B) region of interest (ROI) marked in an interactive manner

X-ray imaging and μCT scan analysis

Directly after retrieval of the samples X-ray imaging was performed by a μCT scanner (Sky Scan 1072, Skyscan NV, Aartselaar, Belgium). Due to the rotation of the objects, radiographs in different positions were taken of each sample. In this way the appropriate area or position for the three-dimensional (3D) reconstruction could be selected and further the X-ray images could be compared with the 3D images.

New bone formation within the defects (n = 4) was evaluated using the same μCT scanner. Specimens were fitted in the middle of a cylindrical sample holder with the coronal aspect of the calvarial bone in a vertical position. High-resolution scanning and cone beam reconstruction were performed with a pixel size of 25.76 μm and slice thickness of 25 μm. The number of slices was set at 20, to cover the entire thickness of the calvarial bone. This was according to the preliminary determined thickness of the skull (20 layers = 0.5 mm), as measured with a caliper and the interactive measurements of the cross-section of the sample. The μCT software was used to make a 3D reconstruction from the obtained set of scans.

Out of the entire 3D data set, a cylindrical ROI with a diameter of 6.18 mm and a height that covered the entire thickness of the calvarial bone. This was according to the preliminary determined thickness of the skull (20 layers = 0.5 mm), as measured with a caliper and the interactive measurements of the cross-section of the sample. The μCT software was used to make a 3D reconstruction from the obtained set of scans.

ROI was placed where the original defect was located since the margins of the original defect were visually recognizable. Within the ROI, the volume of the particles and the volume of the newly formed bone were calculated as a percentage.

The computer program calculated the gray values and the number of voxels with the corresponding gray values in the ROI. A histogram was used to establish which range of gray values should be regarded as bone and which as HA/β-TCP particles. The gray values of the particles and the bone were defined for each sample independently. Applying the threshold according to the histogram, care was taken to eliminate the attenuation of the particles as much as possible since they were visually recognizable. In summary, bone was defined to be in the 129-246 gray value range, whereas the HA/β-TCP particles were regarded to lie in the 0-128 gray value range.

The amount of newly formed bone in the ROI was expressed as relative volume. It was calculated in a percentage as the amount of voxels with bone-related gray values divided by the total number of voxels.

Statistical analysis

One-way Analysis of Variance (ANOVA) was performed on the raw data obtained from μCT and histomorphometry analyses. ANOVA assumes that the data are sampled from populations with identical standard deviations. This assumption was tested using the method of Bartlett. Further, ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption was tested using the Kolmogorov and Smirnov method.

Results

All animals had an uneventful recovery. Their weight remained stable and increased during the implantation period. Further, no infection occurred during that time.

Descriptive histology

Light-microscopic examination of the sections showed new bone formation and almost complete defect closure in all groups after four weeks. Newly formed bone layer was thinner than the surrounding skull bone. The defect borders were still visible. Newly formed bone was characterized by the presence of osteoblasts, osteocytes and an abundance of small blood vessels. The bone had a woven structure without preferred orientation of the collagen fibers in the bone matrix. No signs of bone remodeling or presence of osteoclast-like cells were seen, neither were signs of inflammation observed.

Despite the decalcification procedure, the HA/β-TCP particles could be easily recognized in the skull defects because a circular space was left for the area that was previously occupied by the particles. Analysis revealed that frequently the HA/β-TCP particles were found to be encapsulated by a thin fibrous tissue layer, while embedded in a bone matrix. Occasionally, particles were also surrounded directly by bone (Fig. 3). At the defect borders, bone had bridged the gap between the defect border and the inserted HA/β-TCP
particles.
Overall, no differences in morphology or appearance of newly formed bone were observed among the various groups.

**Figure 3:** HA/TCP-β particles surrounded by thin fibrous tissue layer and embedded in bone, H&E staining
A) 20x magnification    B) 40x magnification

**Histomorphometry**
Histological scoring confirmed the subjective evaluation. We observed that two defects of the empty defect group, three of the particle group, two of the particles + PRP group and two of the particle + PRP gel group were completely closed. On average the defect closure was about 75% for all groups (Fig. 4). Statistical testing showed that no significant differences existed among the groups (p > 0.05). Figure 4 also shows the data of the scoring for bone bridging at the defect borders.

**Figure 4:** Defect closure and bridging at the defect border

Some examples for bridging at the defect borders are presented at Figure 5. Statistical testing again did not reveal the presence of significant differences among the various defect groups.

**Figure 5:** Bridging at the defect border, 2.5x magnification, H&E staining
A) score 0-no bridging    B) score 1-bridging at one defect border    C) score 2-bridging at two defect borders

![Figure 5](image)

Results of the measurements of bone fill percentage in the various defects are presented in Figure 6. The amount of bone formation ranged from 61% to 75%. Statistical testing could not reveal any difference in bone fill percentage between the various groups.

**Figure 6:** Histomorphometric and micro-CT measurements of the amount of the newly formed bone within the defect

![Figure 6](image)

**X-ray imaging and microCT analysis**
Bone formation in the skull defects was also imaged with microCT. In the empty defect new bone formation could be observed clearly on radiographs as well as on 3D models. However, the original drill defect was still visible. In the particle-filled defects, the particles were observed uniformly distributed throughout the defect (Fig. 7). The particles were maintained very well in the skull defect. Only occasionally, some particles were
observed outside the defect. On the 3D images bone formation was especially prominent at the bottom of the particles. Bone had also penetrated in between the particles and resulted in almost complete 3D closure of the defect. Figure 6 shows the quantitative data for the bone volume percentage in the various defects. As it can be seen, the amount of newly formed bone in the ROI expressed as relative volume ranged from 34% (empty defects) to 39% (HA/ß-TCP + PRP group). No significant differences were observed among the groups.

Discussion

The aim of this study was to investigate the effect of PRP on bone regeneration in combination with an osteoconductive material. Fisher 344 rats were animals of choice in this experiment, because they are syngenetic. Therefore, the requirement of PRP as an autologous product is fulfilled and the risk of having false-negative results because of an immune reaction is minimized (Marx 2004). We also managed to achieve in the prepared PRP a 3-fold increase of platelets above the baseline, which is in correspondence with the concentration of platelets obtained by one of the FDA approved devices (3i PCCS, 3i Implant Innovations, West Palm Beach, FL, USA) (Marx 2004).

From the literature (Schmitz & Hollinger 1986), it is known that in rat calvaria the critical-sized defect is 8 mm in diameter after 12 weeks of implantation. On the other hand, 5 mm defects were also described as critical-sized in testing bone-regeneration materials (Bosch et al. 1998; Verna et al. 2002). Based on the literature, we supposed that a 6.2 mm cranial defect would be a critical-sized defect for a 4 weeks implantation period in rats. However, the cranial defects that were prepared, despite being 6.2 mm in diameter and grafted for such short implantation period, turned out to be non critical-sized. An explanation for this discrepancy in results can be the age of the used rats. In our study, the rats were young (11-12 weeks). The capacity of immature animals for accelerated regeneration is well known. Besides age, also species may play a role in the rate of the healing process.

After four weeks of implantation, no significant difference in bone formation between all groups was observed, neither histologically nor by μCT. No positive effect on bone formation of PRP could be determined. This is in agreement with various other studies that evaluated the use of PRP in combination with synthetic bone substitute materials (Froum et al. 2002; Fuerst et al. 2003; Wiltfang et al. 2004). For example, Wiltfang et al. (2004) showed no additional benefit when PRP was applied with xenogenic bone substitutes, such as TCP granules (Cerasorb®), bovine spongious blocks (BioOss®) and collagenous sponge (Colloss®), in critical-sized defects in mini pigs for the same implantation period of four weeks. In a human study with sinus floor lift grafts, Froum et al. (2002) found histologically no benefit by the addition of PRP to BioOss®. On the other hand, Kim et al. (2001) grafted rabbit cranial defects with BioOss® with or without PRP, and reported increased bone density in computed tomography scans at one month when PRP was used. Aghaloo et al. (2004), also in the rabbit cranial model, showed histomorphometrically that the addition of PRP significantly increased the percentage of bone formation over that of BioOss® alone for the same time period (4 weeks).

The fact that in this study PRP did not appear to have any beneficial effect on bone regeneration may be due to the selected implantation time and/or the non critical-size of the created skull defects. Platelets and platelet-derived growth factors are known to be effective during the early stage of bone graft healing, as the life span of a platelet in a wound and the period of the direct influence of its GFs is less than 5 days (Choi et al. 2003). A pronounced effect of PRP will, therefore, especially occur during the early stages of bone regeneration. The defects were also non-critical sized with a tendency to spontaneous healing already within four weeks of implantation. This might have prevented PRP to exert its presumed positive influence. The used PRP preparation technique may even influence the final study outcome. The preparation of PRP from a limited amount of rat blood is not a standard procedure. We followed a protocol used in rat experiments in cardiovascular medicine, where PRP is frequently applied (Yang & Fareed 1997). One of the reasons for choosing this particular protocol was the low centrifugal speed. It is known that the centrifugal speed can influence platelet activation and trigger platelet secretary processes in the plasma during the preparation of platelet concentrates (Kim et al. 2001). By applying a lower centrifugal speed, we supposed to have reduced the chance of occurrence of this phenomenon. The protocol was further adjusted to our needs in order to obtain a platelet concentration, which is considered to be sufficient for inducing a positive effect on bone regeneration (Marx 2004). Weibrich et al. (2004), who have extensively studied and compared different devices for producing PRP, demonstrated in an in vivo experiment that PRP has a very limited range of platelet concentrations to exert a positive impact on bone regeneration. Their study
showed that at lower concentration the effect is suboptimal, while higher concentrations might have an inhibitory effect. Therefore, we suppose that with the protocol used for the production of PRP, this intermediate therapeutic level of platelet concentration and hence, growth factor release was achieved.

The results showed no difference between the effects of PRP liquid or gel on bone formation. Apparently, the only function of thrombin and calcium chloride is to initiate the coagulation process. Still, we have to emphasize that PRP gel increased the handling capacity of the graft because it made the HA/β-TCP particles stick together. However, the whole blood in combination with the HA/β-TCP particles also enabled easier handling of the graft, as it made the particles to stay in place.

Considering the biphasic ceramic (60% HA and 40% β-TCP) that was used, it proved to be osteoconductive. Although the particles did not have an additional favorable effect, they also did not hamper bone formation. This confirms the osteogenic nature of this material and warrants further investigation to the clinical efficacy in critical-sized defects.

As to the methods of evaluation, μCT scanning revealed its great potential in the field of bone research. Although it cannot replace the conventional histology, it promises to be one of the most reliable tools for the estimation of bone ingrowth. It has several advantages, such as it is a non-invasive method, specimens do not need special preparation in staining, embedding and cutting, and it is less time consuming. μCT also provides us with a larger number of slices than are usually used for histomorphometry (in our study 3 versus 20). That explains the higher precision of results and the higher chance on statistically significance compared with histomorphometry (Bernhardt et al. 2004). The calculated volume fraction of bone is based on the true spatial distribution of the material as opposed to extrapolating a volume-based (3D) quantity from a limited set of surface-based (2D) measurements (Verna et al. 2002). In this study, the discrepancy between the histological sections and the 3D images became very obvious. It turned out that the real bone formation could be evaluated only in the 3D model as it presented the complete spatial distribution of the newly formed bone. The 3D image also made it possible to follow the direction of the bone formation over the total defect area, which is especially relevant when particles were applied. In this way we can distinguish whether the particles are lying on the top of the defect or are embedded in bone. All this confirms that 3D images are the most reliable source of information, although there are still problems to be solved with this technique. Some of the difficulties that we encountered were related to the method of calibration and the elimination of the attenuation of the particles. We relied mainly on the histogram defined for each sample independently in order to determine the gray value range of the particles and the bone. This might be regarded as a subjective calibration, rather than an objective one, which is usually performed by means of a phantom model.

In summary, our experiment revealed that PRP had no effect on bone formation for the selected implantation time, independent whether it was applied in the form of a liquid or gel. There was also no difference between PRP liquid and PRP gel, except for the beneficial effect of the gel on the ease of handling of the graft. Dense HA/β-TCP (60%/40%) particles, with a size of 300 - 500 μm proved to be osteoconductive. Further investigations are necessary to elucidate the influence of PRP on osseous regeneration, especially in the early stages of bone healing.

Acknowledgement

We would like to thank the following persons for their contributions to this paper. Cees Verhagen and Joyce Curvers, Sanquin Bloodbank, Nijmegen for providing us the PRP protocol. Dolf Lubbers, Department of Hematology, UMC Nijmegen for counting the platelets with a hematology analyzer. Finally, we would like to thank Natasja van Dijk for her help with the histology. Further, this research was supported by a Huygens grant – programme 2003-2004.
References


Chapter 5

Bone Regenerative Properties of Rat, Goat and Human Platelet-Rich Plasma in Combination with Allograft and Autograft in an Immunodeficient Rat Model

Adelina S. Plachokova, Juliette van den Dolder, Jeroen J. P. van den Beucken, John A. Jansen

Bone, submitted
Introduction

The effect of platelet-rich plasma (PRP) on bone regeneration has been studied extensively since it was first introduced in 1998 by Marx for maxillo-facial reconstructions [1]. Defined as an “autologous concentration of platelets in a small volume of plasma”[2], PRP is considered to be a rich source of growth factors (GFs) as present in the alpha-(α-) granules of blood platelets, i.e. Platelet Derived Growth Factor (PDGF) in its three isoforms (αα, ββ, αβ), Transforming Growth Factor (TGF) in its two isoforms (TGF-β1 and TGF-β2), Vascular Endothelial Growth Factor (VEGF), Epithelial Growth Factor (EGF), Insulin-like Growth Factor 1 (IGF-1) and basic Fibroblast Growth Factor (bFGF), as well as three blood proteins known to act as cell adhesion molecules (fibrin, fibronectin and vitronectin) [1-4]. GFs released upon the activation of platelets are supposed to bind to the transmembrane receptors of osteoprogenitor cells, endothelial cells, and mesenchymal stem cells, and subsequently influence the bone regeneration process through a variety of pathways. The basic hypothesis of the addition of PRP to bone grafts is that high concentrations of platelets in a bony wound will increase initially the local concentration of GFs and subsequently enhance the early bone healing response. In a later stage, the direct influence of PRP will decrease and physiological mechanisms of bone repair will continue to work at an accelerated level [5]. In addition to the present GFs, PRP can influence the bone healing due to its cohesive and adhesive nature. Used in the form of a gel, PRP improves the handling capacity of a graft material with which it is combined, by facilitating graft placement and stability. These features of PRP enable better space maintenance and accelerated bone regeneration.

Although all the above mentioned suggests a huge potential of PRP for the treatment of bone defects, experimental as well as clinical trials are not consistent regarding the impact of PRP on bone healing [6-14]. Moreover, systematic reviews on the topic of PRP can not yet provide solid evidence for the efficacy of PRP application in bone regeneration and augmentation procedures [15,16]. In fact, currently, a lot of confusion exists around the efficacy of PRP treatment in various studies. The contradictory reported PRP treatment outcomes can be due to several reasons, including differences in experimental (animal, human) and bone defect models (critical-, non-critical-sized defects), differences in PRP biology among species, differences in PRP preparation techniques among studies, differences in bone grafting materials combined with PRP and differences in investigated time points. In view of this, the current study attempts to give more insight into the reasons for this discrepancy and to explain the confusion in the literature by exploring the osteogenic capacity of PRP. The aims of this study are:

1. to examine and compare the bone regenerative properties of PRP derived from different species (rat, goat and human) in combination with a synthetic bone substitute;
2. to compare the bone regenerative properties of human bone particles (human bone graft) versus human bone particles (human bone graft) combined with human PRP;
3. to compare the bone regenerative properties of human bone particles (human bone graft) combined with human PRP versus a synthetic bone substitute combined with human PRP.

For this purpose, PRPs and the various materials were installed for implantation periods of 2 and 4 weeks in critical-sized defects (CSDs) as created in the cranium of immunodeficient rats.

Materials and Methods

**PRP preparation**

Rat PRP (rPRP) was prepared from blood of syngenetic rats according to methods described in previous studies [6,7]. Briefly, the whole blood of four male Fisher 344 rats was drawn preoperatively via cardiac puncture and subsequently centrifuged at 800 rpm for 15 minutes (at 25°C).

Goat PRP (gPRP) was supplied by the Sanquin Blood bank Nijmegen. Briefly, one day before surgery, 250 cm³ venous blood was withdrawn from a Saanen goat and centrifuged at a transfusion laboratory (Sanquin, Nijmegen, the Netherlands) in various cycles to result in goat PRP.

Human PRP (hPRP) was prepared and supplied by the Sanquin Blood bank, Nijmegen. About 500 ml autologous blood of a healthy donor was withdrawn in 70 ml of anticoagulants (citrate-phosphate-dextrose [CPD]) and subsequently cooled to about 22°C. Within 24 hours of extraction, the blood was separated through centrifugation into erythrocytes, a buffy coat (leukocytes and thrombocytes) and plasma. From the buffy coat the leukocytes were removed through filtration, and the isolated fraction of platelets was human PRP.

**PRP platelet count**

To obtain information on the increase in platelet concentration and the final concentration of platelets in the PRP of the different species, both whole blood and prepared PRP were subjected to platelet counts. Platelet counts were performed using a hematology analyzer (Advia 120, Bayer B.V., Mijdrecht, the Netherlands).

**Bone grafting materials**

**Synthetic bone substitute**

Biphasic calcium phosphate ceramic in form of dense hydroxyapatite/beta-tricalcium phosphate (HA/TCP, 60%/40%) particles with a size of 300-500 μm (Camceram, CAM Implants, Leiden, the Netherlands) were used as synthetic bone substitute.
Human bone graft

Human bone (HB), harvested from a femur during orthopedic surgery, was supplied by the Sanquin Blood bank Nijmegen, with the approval of the Ethical committee of the Radboud University Nijmegen Medical Center. From it, cortico-cancellous bone graft was obtained by means of trephine drill and ground in a bone-mill (Leibinger®, Freiburg, Germany) into small particles, each portion weighing 1 gram.

PRPs activation

Prior to application at the surgical site, all PRP preparations, i.e. rPRP, gPRP and hPRP, were activated by the addition of 10% calcium chloride solution and bovine thrombin (300 IU) (Fibriquik™ Thrombin, BioMerieux Inc., Durham, NC, USA). For each defect, 150 μl PRP was used and combined with 1 gram of the bone graft material (HA/TCP or HB) before activation.

Surgical procedure

Seventy-two healthy, skeletally mature, male, nude Charles River rats (immunodeficient), with an average weight of 250 g, were used in this study. National guidelines for care and use of laboratory animals were observed, and the study protocol was approved by the Animal Ethical committee of the Radboud University Nijmegen Medical Center. Each rat received one implant, inserted into an 8 mm wide circular cranial critical-sized defect (CSD) [17]. In total, six different experimental groups were assigned for evaluation periods of 2 and 4 weeks (Table 1).

For preparation of the CSDs, anesthesia was induced with 4% Isoflurane by a non-rebreather mask and maintained with 2% Isoflurane, 0.4% N₂O and 0.4% O₂ during surgery. The animals were premedicated by an intramuscular injection of Fentanyl (2.7 ml/kg) to reduce the peroperative pain, and a subcutaneous injection of Buprenorphine (150 μg/kg) was applied to reduce the postoperative pain. Before surgery, the rat cranium was shaved and swabbed with povidone-iodine. A medial sagittal incision extending from the nasofrontal area to the occipital protuberance was made and soft tissues were sharply dissected to visualize the cranial peristeam. Subsequently, the periosteum was undermined and reflected, exposing the parietal bones. A hollow trephine drill (ACE Dental Implant System, Brockton, MA, USA) with an outer diameter of 8 mm in a dental hand piece was used to create a full thickness CSD into the dorsal part of the parietal bone. During surgery care was taken not to damage the dura mater or to puncture the superior sagittal sinus. After insertion of the implants, periosteum and overlying skin were closed in separate layers with 5-0 and 4-0 Vicryl resorbable sutures, respectively.

Histological preparation

Animals were euthanized at 2 and 4 weeks after surgery by suffocation with carbon dioxide. The skin was dissected and the defect sites were removed along with the surrounding bone and soft tissues, and fixed in 10% neutral formalin. All specimens (n = 6 per material and evaluation time) were dehydrated in gradual series of alcohol and embedded in polymethylmethacrylate (PMMA). After polymerization, sections were prepared using a modified diamond-blade sawing microtome technique (Leica Microsystems GmbH, Wetzlar, Germany). Obtained sections were approximately 10 μm thick and stained with methylene blue and basic fuchsin.

The light microscopical evaluation of all sections was done using an optical microscope (Leica BW, Rijswijk, the Netherlands), and consisted of a complete morphological qualitative and quantitative description of the tissue response. For evaluation of the bone healing process a modified histological scoring system was developed (Table 2A) and a comprehensive assessment was performed on three sections per cranial specimen. Each section was assigned a score, and from the scores of all sections in one group the average score of the group was determined.
**Micro-CT (μCT) scans analysis**

Computed tomography of randomly chosen samples (n = 4) was performed using a μCT scanner with very high spatial resolution (5 μm corresponding to near 1 x 10^8 cubic mm voxel size) (Sky Scan 1072, SkyScan NV, Aartselaar, Belgium). Tomographic data were acquired by cone-beam acquisition of the specimens (100kV/98μA, 25.92 x magnification, 6.5 s exposure, rotation 180 degrees) and reconstructed in 512 x 512 pixel matrices by a convolution-back projection procedure. For each sample, a total of 336 micro-tomographic slices were gained using a slice increment of 25 μm in order to scan the whole defect (8 mm) and the surrounding bone. Measurements were stored in 3D image arrays of high resolution (1024 x 1024).

From the μCT scan datasets, 3D models were built for visualization and morphometric analysis. From the 3D models, the degree of bone union was defined following the method developed by Hedberg [18] (Table 2B). For the morphometric analysis, database of 320 axial projection skull images was used per sample (n = 4). Further, the same approach as described in a previous study by our group [7] was applied. Briefly, the defect with the newly formed bone inside was selected as a region of interest (ROI), which by auto-interpolation of layers became volume of interest (VOI). Within this VOI, the volume of the HA/TCP particles and the volume of the newly formed bone (defined as bone fill %) were calculated. For the HB-groups, since it was not possible to distinguish between newly and grafted bone due to their similar density, the total bone volume inside the defect was calculated and described as bone volume %. For all 3D-measurements grayscale images were used, which were binarised based on histogram calculations (256 grayscale, bone range: 64-225; HA/TCP particles range: 0-63). The areas presented in white were identified as image objects and all those presented in black were considered as background. The volume of the newly formed bone (bone fill in mm^3) or total bone volume (as defined for HB-groups) was the total volume of binarised objects within the VOI. The percentage of the newly formed bone (bone fill %) or total bone volume % was the proportion of the VOI occupied by binarised solid objects.

**Statistical analysis**

GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses of % bone fill and % bone volume data as obtained by μCT analyses (i.e.). One-way Analysis of Variance (ANOVA) combined with Tukey-Kramer Multiple Comparisons Test was performed. ANOVA assumes that the data are sampled from populations with identical standard deviations. This assumption was tested using the method of Bartlett. Further, ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption was tested using the method

---

### Table 2: Scoring Guides

**A. Histological semi-quantitative scoring guide**

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct bone to implant contact without fibrous tissue interfaces at both defect edges</td>
<td>2</td>
</tr>
<tr>
<td>Direct bone to implant contact at only one side, fibrous tissue at the other defect edge</td>
<td>1</td>
</tr>
<tr>
<td>No direct bone to implant contact (fibrous tissue at both defect edges)</td>
<td>0</td>
</tr>
</tbody>
</table>

**B. Hard tissue response in between the particles/ bone fillers abbr. (Tissue res)**

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue in between the particles is mostly bone</td>
<td>3</td>
</tr>
<tr>
<td>Some bone with fibrous tissue</td>
<td>2</td>
</tr>
<tr>
<td>Mostly fibrous tissue</td>
<td>1</td>
</tr>
<tr>
<td>No bone present</td>
<td>0</td>
</tr>
</tbody>
</table>

**III. Bone fill in vertical direction abbr. (Bone fill)**

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newly formed bone is filling the defect through and through</td>
<td>3</td>
</tr>
<tr>
<td>Newly formed bone is filling the defect half (1/2) of its height</td>
<td>2</td>
</tr>
<tr>
<td>Newly formed bone is filling the defect 1/3 of its height</td>
<td>1</td>
</tr>
</tbody>
</table>

**IV. Defect closure abbr. (D closure)**

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>75-100% consists of bone</td>
<td>4</td>
</tr>
<tr>
<td>50-74% consists of bone</td>
<td>3</td>
</tr>
<tr>
<td>25-49% consists of bone</td>
<td>2</td>
</tr>
<tr>
<td>1-24% consists of bone</td>
<td>1</td>
</tr>
<tr>
<td>0-1% consists of bone</td>
<td>0</td>
</tr>
</tbody>
</table>

**V. Direction of bone formation (sources of newly formed bone) abbr. (Dir of NFB)**

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone formation from the periosteum, dura side and defect edges</td>
<td>3</td>
</tr>
<tr>
<td>Bone formation from the dura side/particles and the defect edges</td>
<td>2</td>
</tr>
<tr>
<td>Bone formation only from the defect edges</td>
<td>1</td>
</tr>
</tbody>
</table>

**B. Micro-CT scoring guide for degree of bone union (according to Hedberg et al. 2005)**

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bony bridging at longest point of the defect</td>
<td>4</td>
</tr>
<tr>
<td>Bony bridging over partial length of the defect</td>
<td>3</td>
</tr>
<tr>
<td>Bony bridging at defect edges</td>
<td>2</td>
</tr>
<tr>
<td>A few bony spicules dispersed through the defect</td>
<td>1</td>
</tr>
</tbody>
</table>
of Kolmogorov and Smirnov. Statistical significance was set at p < 0.05. Statistical analyses on the histomorphometrical and μCT degree of bone union data were performed using Poisson regression, as data were not normally distributed. Poisson regression was used to elucidate group and time effects. Statistical level of significance was set at p < 0.05.

Results

General observations
Out of 72 rats used in this study, 4 died during surgery due to severe bleeding caused by the rupture of the sagittal sinus (Table 1). The remaining 68 rats had an uneventful recovery and were housed separately to reduce the risk of infection occurrence. National guidelines for care and use of laboratory animals were rigorously followed. No clinical signs of infection or inflammatory response were seen, and all rats gained weight during the implantation period.

PRP preparations
Platelet counts in rat, goat and human PRPs showed substantial increase above the baseline number of platelets in the whole blood of the species as presented in Table 3.

### Table 3: Platelet count in PRPs

<table>
<thead>
<tr>
<th>Species</th>
<th>Whole blood</th>
<th>PRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>1099.10^9/l</td>
<td>3172.10^9/l</td>
</tr>
<tr>
<td>Goat</td>
<td>171.10^9/l</td>
<td>800.10^9/l</td>
</tr>
<tr>
<td>Human</td>
<td>244.10^9/l</td>
<td>2235.10^9/l</td>
</tr>
</tbody>
</table>

Histological descriptive and quantitative evaluation

Two weeks results
After 2 weeks of implantation, only minor bone formation was seen in all groups, which occurred mainly at the defect edges (Figure 1).

![Figure 1: Light micrographs of PMMA embedded specimens showing bone formation in all groups after 2 and 4 weeks (1.6 x magnification), bar = 625 μm](image)

Occasionally, some bone ingrowth from the dura site was detected in both graft alone groups (HA/TCP and HB) and both hPRP groups (HA/TCP+hPRP, HB+hPRP). Within the defect, bone grafts (HA/TCP and HB) in all groups with and without PRP were found completely surrounded by fibrous tissue (Figure 2), and occasionally, start of guided bone formation around the particles was noticed, without any preference for a particular group. At the defect edges, direct bone to implant contact without any fibrous tissue interface was found mainly in the groups with HB (HB and HB+hPRP). In contrast, only half of the sections of HA/TCP and HA/TCP combined with different PRPs showed direct bone to implant contact at one defect border.

![Figure 2: Histological sections of PMMA embedded specimens showing tissue response in between the particles of the human bone graft (HB) and the allograft (HA/TCP) (20 x magnification), bar = 50 μm](image)

The data obtained with the histological scoring system are depicted in Figure 3A. Statistical analyses indicated that after 2 weeks of implantation, rat, goat and human PRP combined with HA/TCP showed almost comparable scores in each category related to the bone healing process, such as hard tissue response at bone-implant interface, hard tissue response in between the particles/ bone fillers, bone fill in vertical direction, defect closure and direction of bone formation. Overall, defects filled with hPRP and bone particles (HB+hPRP) showed slightly better scores than those filled with HB alone. However, this difference was not statistically significant. hPRP in combination with HB (HB+hPRP) showed comparable scores to hPRP in combination with HA/TCP (HA/TCP+hPRP) for most of the parameters (Figure 3A). Only for the defect closure, the scores of HB+hPRP were significantly higher than those of HA/TCP+hPRP group (p < 0.05).
Four weeks results

After 4 weeks of implantation, light microscopical analysis demonstrated clear bone formation at the defect edges and at the dura site in all groups (Figure 1). Only one specimen of the HA/TCP+rPRP group showed the start of new bone formation from the periosteum. Generally, at higher magnification no differences in morphology of the newly formed bone were observed among the various groups. Newly formed bone in all slides appeared as a woven structure without preferred orientation of the collagen fibers in the bone matrix and an abundance of osteoblasts and small blood vessels was observed. No signs of bone remodeling or presence of osteoclast-like cells were seen, neither were signs of inflammation present. However, the bone formation process appeared to be more active in the HB group, where newly formed bone was bridging the human bone particles (Figure 2). Bone bridging was found at both defect borders in all groups, except for the HA/TCP+gPRP, where in half of the specimens a connective tissue layer still filled the gap between the defect edge and the inserted HA/TCP particles. Tissue formation in between the graft materials (HA/TCP and HB) with and without PRP showed hardly any difference among all groups, and consisted of some bone and fibrous tissue. For all groups with HA/TCP particles with and without PRP, newly formed bone was guided by their outer surfaces (Figure 2). This guidance was at best to be seen in the HA/TCP and HA/TCP+hPRP group. Further, HA/TCP, HA/TCP+hPRP, HB and HB+hPRP particulates were directly embedded in bone without an intervening fibrous tissue layer. In contrast, in the HA/TCP+gPRP and HA/TCP+rPRP groups, bone substitute particles were still surrounded by a thin fibrous tissue layer. Although no specific staining for blood vessels was performed, gross evaluation revealed comparable neovascularization in all groups.

The data obtained with the histological scoring system are depicted in Figure 3B. Statistical analyses indicated that after 4 weeks of implantation, rat, goat, and human PRP combined with HA/TCP, and HA/TCP alone showed similar scores for each parameter. Again, scores of HB+hPRP were comparable to those of HA/TCP+hPRP, except for defect closure, which was significantly higher for the HB+hPRP group (p < 0.05). Defects filled with HB+hPRP showed comparable scores with HB alone. Results of HB+hPRP were slightly superior over the results of HA/TCP+hPRP, though not significantly different.

In addition, all groups showed a significant increase from 2 to 4 weeks for the following parameters: hard tissue response at bone-implant interface (p < 0.001), direction of new bone formation (p < 0.05) and hard tissue response in between the particles/bone fillers (p < 0.001).

μCT analysis

Degree of bone union

Two weeks results

3D models demonstrated bone formation in all groups after 2 weeks of implantation. For the HA/TCP, HA/TCP+gPRP and HA/TCP+rPRP, the degree of bone union was in the form of a few bony spicules dispersed through the defect, whereas for the HA/TCP+hPRP group bony bridging at the defect edges was observed. All data dealing with the degree of bone union are presented in Figure 4. Scores of HB+hPRP group exceeded in general the scores of HB group and HA/TCP+hPRP, however, this was not significant. Statistical analyses indicated a significant difference for degree of bone union in favour of HB+hPRP compared to HA/TCP, HA/TCP+gPRP, and HA/TCP+rPRP groups (p < 0.01).
3D reconstructions revealed that the addition of hPRP to the HB particles resulted in bony bridging over the partial length of the defect, whereas when HB particles were applied alone, bony bridging was limited to the defect edges.

**Four weeks results**

At 4 weeks, of implantation, 3D modeling and statistical analyses demonstrated no change in the degree of bone union in all groups compared to the scores after 2 weeks. Briefly, HB+hPRP showed the highest degree of bone union, which was significant compared to HA/TCP, HA/TCP+gPRP, and HA/TCP+rPRP (p < 0.01). No difference was observed between HA/TCP+hPRP, HB and HB+hPRP.

**Bone Volume**

**Two weeks results**

After 2 weeks, the amount of newly formed bone (% bone fill) was similar for HA/TCP, HA/TCP+gPRP and HA/TCP+rPRP, i.e. ± 7% (Figure 5A), while the HA/TCP+hPRP showed significantly more bone fill, i.e. 14% ± 2 (p < 0.001). The total bone volume for HB+hPRP (18% ± 2) was significantly higher than the bone volume for HB (14% ± 1) (p < 0.01). (Figure 5B).

**Discussion**

This study was performed to provide an explanation for the existing confusion in the literature regarding the efficacy of PRP treatment, as well as to give more insight into the effect of PRP on bone regeneration. All PRP preparations as carried out in this study met the recommendations in terms of preparation technique (centrifugation speed, force and time) and platelet concentration. In view of the correlation between platelet count and growth factor levels [2], it is likely that the amounts of GFs as released from rat and goat PRP were not comparable with those of human PRP. This suggestion is supported by the findings of Trowbridge [19] that rat mean platelet count is significantly higher than human, whereas rat mean platelet volume is significantly lower than human. In view of the lower rat platelet volume and the fact that GFs are stored in plate-
histomorphometric examinations showed no effect of goat PRP after 1, 2, 6, and 12 weeks of implantation. The authors refer to contradictory information about the baseline concentrations of platelets in goats [24-26]. As a consequence, it cannot be excluded that their concentration of PRP was just too low to induce any effect at all. Since in the current study goat PRP was prepared using a similar protocol, it can be speculated that the goat PRP in our experiment was also not sufficiently concentrated.

Our histological and μCT data revealed an early beneficial effect of human PRP on bone healing. These findings support the suggested action mode of PRP. The active secretion of GFs from the platelets begins within 10 min after blood clotting and more than 95% of the presynthesized GFs in the α-granules of the platelets are secreted within 1 hour. After the initial burst release of PRP-related GFs, platelets synthesize and secrete additional GFs for the remaining 10 days of their life span [2]. Therefore, PRP is supposed to affect the early bone healing, rather than late bone formation.

The observed enhanced effect of human PRP, when combined with human bone graft material compared to a combination with a synthetic bone substitute, can also be explained by the mechanism of action of PRP. According to Marx, PRP is postulated to exert its effect on living cells [1]. Consequently, when PRP is used together with synthetic, non-cellular bone substitutes less promotion of bone formation compared to the use with bone graft material could be expected. The beneficial effect of PRP applied in combination with a synthetic bone substitute, will depend on the amount of resident osteoprogenitor cells at the bone defect site. Occasionally, it is supposed that osteoconductive materials can obscure the true effect of PRP [21,27].

The use of various analytical techniques, like histology and μCT, to evaluate the process of bone formation is considered to provide the most complete and reliable data [18]. A morphological analysis of μCT 3D models gives more comprehensive and reliable information than histological sections alone. Histological sections are 2D images and always limited in number (3 sections per sample vs. 320 sections per sample for the μCT), which partially explains the slight differences between the current histological and μCT results. In addition, μCT facilitates the histomorphometrical analysis of bone fill and bone ingrowth [6]. In view of this, it has to be noticed that the histological “bone fill in vertical direction” is a morphometric parameter indicating the appearance of the newly formed bone, whereas the measured μCT “bone fill” is a volumetric parameter demonstrating the amount of the newly formed bone. Evidently, in the current study, the correlation between the histological and μCT results were proven once again. The results on defect closure, a histological 2D parameter, coincided with the results for both bone union and bone volume (3D μCT parameters).

Despite the beneficial effect of human PRP in combination with human bone graft, the amount of bone formation was still limited and less than expected for “autologous” material. Probably, this is due to the source of the graft material, as the human bone...
was harvested during surgery for installation of a hip implant. Patients subjected to this kind of surgical intervention are prone to poor bone quality in the surgical area, since the most common indication for total hip replacement surgery is osteoarthritis. Osteoarthritis together with osteoporosis are known as age-associated ‘degenerative diseases’. It is known that with increasing age there is a relative decline in trophic factors (e.g. oestrogen, IGF-1, vitamin D) favouring local expression of molecules (interleukins, TNFα), which decrease osteoblast activity, increase osteoclast activity, and reduce the differentiation potential of bone marrow stem cells [28]. Therefore, it can be speculated that bone graft harvested from a patient suffering from age-associated degenerative disease possesses inferior osteogenic properties. In addition, it can be suggested that the size of the particles also plays a role in the observed limited effect. It is known that too large particles hinder adaptation of the graft material to the defect site. Since in the μCT 3D models the particles appeared to be not so uniformly distributed as the HA/TCP, an inappropriate size of the particles for this particular type of defect cannot be excluded.

Within the limitations of this study (in terms of animal species and used PRP preparation technique), the PRP derived from the used animal species was shown to have no effect on bone regeneration neither at early nor at late time points. In contrast, human PRP seemed to have a beneficial effect on bone regeneration. These findings together with observation that the majority of animal studies in the literature fail to prove an osteopromotive effect of PRP, raises the question to what extent animal studies can be used to draw conclusions on the potential of human PRP. In addition, there is serious doubt that animal PRP and its preparation is completely comparable to human PRP. On the other hand, some evidence exists that PRP has a beneficial effect on maxillo-facial bone regeneration, but this proof is weak [15,16, 29]. In view of this, it has to be recommended that future efforts be focused on well-designed human randomized-controlled clinical trials to provide complete support for the clinical application of PRP instead of the continuation of animal studies.

Conclusions

In summary, our study showed that rat and goat PRP had no effect on bone formation, neither at early nor at late time points. Human PRP combined with human bone graft material resulted in an increased osteogenic effect over bone graft material alone after 2 weeks of implantation. At 4 weeks, this additional effect of human PRP was not observed. Finally, the combination of human PRP with human bone graft material seems to be more effective than the use of human PRP with a synthetic calcium-phosphate based bone substitute.

Acknowledgements

We would like to thank the following people for their contribution to this paper. Cees Verhagen and Joyce Curvers, Sanquin Bloodbank, Nijmegen for providing the PRP protocol. Dolf Lubbers, Department of Hematology, UMC Nijmegen for counting the platelets with a hematology analyzer. Natasja van Dijk for sectioning and histology, Vincent Cuijpers for μCT scanning and Jan Mulder for his help for the statistical analyses.
References:

Chapter 6

Bone Regenerative Properties of Injectable PLGA/CaP Composite with TGF-β1 in a Rat Augmentation Model

Adelina S. Plachokova, Dennis P. Link, Juliette van den Dolder, Jeroen J. P. van den Beucken, John A. Jansen.

Journal of Tissue Engineering and Regenerative Medicine 2007
Nov-Dec; 1(6): 457-64
Introduction

Autogenous bone is still considered to be the “golden standard” for bone augmentation. However, due to the known limitations of autogenous bone grafting, such as donor site morbidity and limited supply, increasing efforts focus on the development of bone substitutes. In order to create a bone graft, which is comparable or superior to bone, allografts are combined with osteoinductive substances, such as growth factors. Transforming growth factor-β1 (TGF-β1) is a member of TGF-β superfamily, which regulates key aspects in bone metabolism (Massagué J. 1990). It affects osteoblast differentiation, matrix formation, and mineralization. Furthermore, it exerts its functions both during embryogenesis and in adult organisms, orchestrating complex phenomena such as inflammation and tissue repair (Roberts and Sporn 1993). Studies have shown that TGF-β1 supplied to a variety of biomaterials is able to enhance bone formation in vivo, depending on concentration and application method (Okuda et al., 1995; Sumner et al., 1995; Lind et al., 1996). Consequently, TGF-β1 is an appealing factor for use in clinical procedures for bone augmentation.

As mentioned above, various materials have already been used for local delivery of TGF-β1, including calcium phosphate (CaP) ceramics (Okuda et al., 1995; Sumner et al., 1995; Lind et al., 1996). This material has been selected for bone replacement and augmentation due to its excellent bone behavior. The shortcomings of the prefabricated CaP ceramics, such as lack of shaping and difficult delivery to the defects, have been overcome by the development of injectable CaP cements (Ooms et al., 2005). Injectable self-setting cement can fill macroscopic as well as microscopic bone defects. Cements of this kind may be resorbed, allowing gradual replacement by host bone (Jansen et al., 1995). To promote degradation and tissue ingrowth injectable CaP cements have been combined with PLGA microspheres (Ruhe et al., 2003). Additionally, these degradable PLGA microspheres provide a method for delivery of bone formation promoting proteins, like TGF-β1. Such an injectable composite has been developed by our group and comprehensively studied in vitro and in vivo (Ruhe et al., 2003; 2005; 2007). However, the direct mode of application in a bone augmentation model has not been evaluated yet.

In view of the above mentioned, the aim of this study was to examine the bone augmentation properties of an injectable PLGA/CaP composite and to evaluate the additional effect of loading the composite with TGF-β1 in a rat model.

Materials and Methods

Materials

The calcium phosphate cement Calcibon® (Biomet Merck, Darmstadt, Germany) was used in this study. The cement powder consisted of 62.5% α-TCP (alpha tri-calcium phosphate), 26.8% CaHPO₄, 8.9% CaCO₃, and 1.8% PHA (precipitated hydroxyapatite). An aqueous solution of 1% NaHPO₄ was used as a liquid component with a liquid/powder ratio of 0.35 ml/g. Low-molecular weight poly (D, L-lactic-co-glycolic acid) (PLGA) Purasorb® was provided by Purac, Gorinchem, the Netherlands. Human rh-TGF-β1 was supplied by R&D Systems, Abingdon, UK.

Preparation of PLGA microspheres

PLGA microspheres were prepared using a water-in-oil-in-water (w/o/w)-double emulsion solvent evaporation technique, as described previously by Ruhe et al. (Ruhe et al., 2003). Briefly, 1.4 g of low molecular weight (LMW) PLGA was dissolved in 2 ml of dichloromethane (DCM) inside a 50 ml poly propylene (PP) tube. 500 ml demineralized water (ddH2O) was added while vortexing vigorously for 1 min, subsequently adding 6 ml of a 0.3% poly-vinylalcohol (PVA) solution. Vortexing was continued for another 1 min. The content of the 50 ml tube was transferred to a stirred 1000 ml beaker and another 394 ml of 0.3% PVA was added slowly. This was directly followed by adding 400 ml of a 2% isopropyl alcohol (IPA) solution. The suspension was stirred for 1 hr. The spheres were allowed to settle for 15 min and the solution was decanted. The suspension left was centrifuged, and the clear solution at the top was decanted. Five ml of ddH2O was added, the spheres were washed, centrifuged and the solution was aspirated. Finally the spheres were frozen, freeze-dried for 24 hr and stored under argon at -20°C.

Adsorption of TGF-β1 on PLGA microspheres

One μg of recombinant human TGF-β1 (rh-TGF-β1) was dissolved into 800 μl of 0.1% acetic acid and added to 1.0 g of microspheres, after which the TGF-β1 was freeze-dried onto the microspheres. The entrapment efficiency of the protein was determined by normalizing the amount actually entrapped with the amount added to the fabrication process.

Preparation of PLGA/CaP composites

Composites were made by adding PLGA microspheres to the CaP cement in a weight ratio 20/80 PLGA/CaP cement. The cement powder was sterilized by gamma radiation with 25 kGy (Isotron B.V., Ede, the Netherlands) and the cement liquid was filter-sterilized (0.2 μm filter). Firstly, 256 mg CaP cement powder provided with 64 mg PLGA...
periosteum and overlying skin were closed in separate layers with 5-0 and 4-0 Vicryl material and evaluation time). Anesthesia was induced with 4% Isoflurane by a non-rebreather mask and maintained with 2% Isoflurane, 0.4% N₂O and 0.4% O₂ during surgery. The animals were premedicated by an intramuscular injection of Fentanyl (2.7 ml/kg) to reduce the operative pain, followed by an intramuscular injection of Buprenorphine (150 μg/kg) to reduce the postoperative pain.

Before surgery, the rat cranium was shaved and swabbed by iodine. A medial sagittal incision extending from the nasofrontal area to the occipital protuberance was made on its parietal cranial bone for evaluation periods of 2, 4 and 8 weeks (n = 8 for each material and evaluation time).

Surgical procedure
For the animal experiment, twenty-four healthy, skeletally mature male Wistar rats, with an average weight of 250 g, were used. National guidelines for care and use of laboratory animals were observed, and the Animal Ethical committee of the Radboud University Nijmegen Medical Center approved the study protocol. In total, 48 composite implants were placed; each animal received 2 implants from the same group on its parietal cranial bone for evaluation periods of 2, 4 and 8 weeks (n = 8 for each material and evaluation time).

Anesthesia was induced with 4% Isoflurane by a non-rebreather mask and maintained with 2% Isoflurane, 0.4% N₂O and 0.4% O₂ during surgery. The animals were premedicated by an intramuscular injection of Fentanyl (2.7 ml/kg) to reduce the operative pain, and a subcutaneous injection of Buprenorphine (150 μg/kg) was applied to reduce the postoperative pain.

Before surgery, the rat cranium was shaved and swabbed by iodine. A medial sagittal incision extending from the nasofrontal area to the occipital protuberance was made on its parietal cranial bone for evaluation periods of 2, 4 and 8 weeks (n = 8 for each material and evaluation time).

Histological preparation
The animals were sacrificed 2, 4, and 8 weeks after surgery using an overdose of carbon dioxide. The skin was dissected and the defect sites were removed along with the surrounding bone and soft tissues, and fixed in 10% neutral formalin for one week. Half of the specimens (n = 4 per material and evaluation time) were dehydrated in gradual series of alcohol and embedded in polymethylmethacrylate (PMMA). Before the embedding procedure, samples were cut exactly through the middle of the implants to secure that the microscope sections were made in the area of interest and the same region was analyzed for each sample. After polymerization, sections were prepared using a modified diamond-blade sawing microtome technique (Leica Microsystems GmbH, Wetzlar, Germany). Obtained sections were approximately 10 μm thick and stained with methylene blue and basic fuchsin. The remaining specimens (n = 4 per material and implantation time) were decalcified for 2 weeks in Formical-2000 (Immunodiagnostics & Biotech GmbH, Berlin, Germany) and then embedded in Paraplast paraffin (Klinipath B.V., Duiven, the Netherlands). Sagittal microtome sections were made, 5 μm in thickness, which were stained with hematoxylin-eosin and immunohistochemically using an antibody against CD68 (Macrophilin (Janeway et al., 1999) to detect inflammatory cells including monocytes, macrophages, and neutrophils. Again, before the embedding procedure, samples were cut exactly through the middle of the implants.

The light microscopical evaluation of all sections was done using an optical microscope (Leica BW, Rijswijk, the Netherlands), and consisted of a complete morphological description of the tissue response to different implants as well as of histomorphometrical analysis.

Histomorphometry
For the histomorphometrical analysis, a Leica Qwin Proimage analysis system (Leica BV, Rijswijk, the Netherlands) was used. Sections were digitized at low magnification (2.5 x) and measurements were done on three sections per cranial specimen. All PMMA sections were used for evaluation of bone response and all paraffin sections for assessment of inflammatory reaction.
To quantify the amount of bone augmentation, newly formed bone, as stained and discerned by its woven structure and location, was marked manually and its area was measured. Afterwards, this ROI was subtracted from the whole area of the implant. From these two data, the amount of bone formation (%) was calculated.

In order to measure the contact length between the implants and the bone, lines were drawn manually at the direct interface between the composites and newly formed bone. The sum of these intersections was calculated and interpreted as contact bone/composite length.

Further, the intensity of the inflammatory response around the implants was quantified. For this purpose pictures with higher magnification (i.e. 10x) were used. The presence of macrophages was detected with anti-CD68 staining and subsequently digitized by a computer programme in the region of interest (implant + surrounding tissue; ROI). The areas with positive anti-CD68 staining were given as a percentage (%) of the total ROI.

Statistical analysis

For the statistical analyses, GraphPad InStat program (GraphPad Software, San Diego, CA, USA) was used. One-way Analysis of Variance (ANOVA) was performed on the data obtained from the histomorphometric analyses. ANOVA assumes that the data are sampled from populations with identical standard deviations. This assumption was tested using the method of Bartlett. Further, ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption was tested using the method of Kolmogorov and Smirnov. In addition, a Tukey-Kramer Multiple Comparisons Test was done. Differences were considered to be significant when p < 0.05.

Results

General observations

The injection of the composite into the silicon mold placed on the scratched cranial surface went without complications. The setting time of both loaded and unloaded composites was about 10 min. Setting and handling properties of the composites were influenced by the presence of blood. When the composite was injected on dry, good isolated cranial surfaces the setting time was shorter, the composite was easily shaped, and after removal of the mold the implant was secured more stable. Mixing with blood affected the handling properties of the composites and made them more fragile. In addition, the composites loaded with TGF-β1 appeared to be more fragile after setting than the unloaded composites, as indicated by easy cracking.

During the implantation periods all animals remained in good health. At sacrifice, macroscopic signs of inflammation and adverse reaction were apparent around loaded as well as unloaded implants predominantly after 2 and 4 weeks. After 8 weeks, the signs of inflammation disappeared. Upon retrieval, all implants in both groups were in situ and surrounded by a fibrous capsule. The capsule thickness differed between different time points with a tendency of reduction for the later periods. No differences between the loaded and unloaded composites were observed.

Descriptive histology

Histological examination of PMMA embedded samples revealed new bone formation in both groups at all time points. Newly formed bone was more abundant at 4 and 8 weeks in comparison to its amount at 2 weeks (Figure 2). Bone formation was observed at the defect margins and along the lower part of the cement at the side of the cranium. No bone formation was seen from the periosteum side on the top of the implants, as well as throughout the implant.

Figure 2: Light micrographs of PMMA embedded specimens showing bone augmentation at different time points (2.5x magnification)

At higher magnification, newly formed bone revealed thin trabeculae and large marrow spaces filled with fat tissue and haematopoietic cells. In addition, a direct contact between the composites and newly formed bone, without any fibrous tissue interface, was apparent in both experimental and control groups (Figure 3).

Figure 3: Histological section of PMMA embedded specimen (10x magnification). A) unloaded PLGA/CaP composite B) TGF-β1 loaded PLGA/CaP composite

Guided bone formation is visible with direct contact composite - newly formed bone without any fibrous tissue interface. Dash line in yellow indicates the transition between the newly formed bone and the old bone. OB - old bone, NB - newly formed bone, C – composite.
Furthermore, implants managed to stay in place during all investigation periods. However, the integrity of the composite structure appeared to be affected. Fractures, as observed inside the implants, were filled with fibrous tissue and inflammatory cells. Signs of inflammation were at best visible in the paraffin sections stained for CD68. The inflammatory infiltrate, consisting of macrophages and lymphocytes, surrounded the implants and penetrated into the composite structure. In the course of time a substantial decrease in the inflammatory response was observed in both groups (Figure 4). Together with the decrease in inflammation, a consolidation of the implant fragments and their incorporation in the bone tissue was noticed.

![Fig 4: Light micrographs of paraffin sections stained for CD68 showing the inflammatory response at different time points (2.5x magnification).](image)

Paraffin sections stained with hematoxylin-eosin did not provide any additional information to the PMMA sections.

**Histomorphometry**

The amount of newly formed bone was almost the same for both experimental and control groups after 2 and 4 weeks. At 8 weeks, there was more bone formation in the group of TGF-β1 loaded implants (18.5% ± 3) than in the group of the unloaded ones (7.2% ± 5), which was statistically significant (p < 0.05; Figure 5).

The contact length between the composites and newly formed bone increased in time. At 2 weeks TGF-β1 loaded samples showed significantly better bone contact than unloaded controls (19 mm ± 7 vs. 2.7 mm ± 4 and p < 0.05). After 4 and 8 weeks, no difference in the contact length was observed between the groups (Figure 6).

The amount of inflammation after 2 weeks was substantial for both groups. However, after 4 weeks it decreased to 9.0% ± 2 for the TGF-β1 loaded implants and to 13.3% ± 2 for the controls. This difference between the groups appeared to be statistically significant (p < 0.01). At 8 weeks a minor inflammation was observed in both groups (Figure 7).
Discussion

The present study demonstrates that the used injectable PLGA/CaP (20/80) composite significantly enhanced new bone formation at 8 weeks when compared to the control (i.e. unloaded PLGA/CaP composite). The osteopromotive effect of TGF-β1 has been demonstrated in many studies (Arnaud et al., 1994; Zhou et al., 1995; Sumner et al., 1995; Lind et al., 1996; Beck et al., 1991; 1993; Moxham et al., 1996). There are, however, studies that describe absent or even adverse effect of TGF-β1 on bone formation (Lind et al., 1994; Aspenberg et al., 1996). Since it is known that follow-up period, as well as animal species may play role in this aspect (Janssens et al., 2005), it has been decided to compare the outcomes of the current study with similar experimental studies. Our findings corroborate the results of Vuola et al. (Vuola et al., 2002). In their rat cranial model TGF-β1 loaded coral based implants enhanced bone regeneration, and the amount of newly formed bone was also not significantly higher compared to the controls until the 8th week. The authors suggested that the influence of TGF-β1 on bone formation lasted several weeks and diminished with age. In the study of Vuola et al. 6-8 months old rats, weighing 550-560 g, were used. In our study the experimental animals were younger, yet skeletally mature, and with a weight of approximately 250 g. Further, the concentration of TGF-β1 incorporated in our composites (200 ng) was much lower than that used in the study of Vuola (1, 2 and 25 μg).

The effect of TGF-β1 on bone formation is known to be dose dependent, however it has to do more with an optimal than with a maximal dose (Beck et al., 1993). For example, Blom et al. (Blom et al., 2001) reported enhanced bone formation for the same implantation period with very low concentrations of TGF-β1 (10 ng and 20 ng) incorporated in a similar type of CaP cement. In the current experiment enhanced bone formation was observed also with a lower TGF-β1 dose than applied in similar studies (Okuda et al., 1995; Bosch et al., 1996). Therefore, it can be suggested that the amount of TGF-β1 needed to exert an effect may depend on the type of carrier used.

Delivery systems are of great importance when growth factors are utilized to enhance bone-healing processes (Puolakkainen et al., 1995). In previous studies (Ruhe et al., 2003, 2005) it was demonstrated that: (a) PLGA/CaP composite is a suitable carrier for GFs, and (b) the method of loading GFs on the scaffold (freeze-drying on top of the PLGA microspheres) does not affect their osteoinductive properties. However, the high protein binding affinity of CaP cement must be taken into account. Therefore, it can be hypothesized that after release from the PLGA microspheres, TGF-β1 may have bound to the CaP cement resulting in a delayed release from the composite. This might explain why no increase in bone formation was seen after 4 weeks for TGF-β1 loaded materials compared to the controls. At the moment, it is still unclear whether the TGF-β1 as bound to the CaP cement has maintained its osteopromotive properties. We have to notice that this has to be an issue of investigation.

It has also to be emphasized that the effect of TGF-β1 on bone formation differs among species. In general, studies using rodents and primates as experimental models do not report so favorable outcomes concerning the osteopromotive ability of TGF-β1 compared to studies using dogs (Janssens et al., 2005).

In the current study the release kinetics of the TGF-β1 from the PLGA/CaP composite was not examined. On the other hand, similar designed PLGA/CaP composites were recently used in another study for the delivery of enamel matrix derivate (EMD) (Plachokova et al., In press). An in vitro assay revealed that after 4 weeks only 60% of EMD was released from these scaffolds, and this sustained release continued as observed during extended follow-up periods. In view of this, it can be speculated that such a release pattern is too slow for TGF-β1 to support early bone development. Therefore, the TGF-β1 promoted bone formation after 4 weeks was enhanced but not statistically significant compared to the control. Nevertheless, it must be taken into account that EMD and TGF-β1 are completely different proteins. Additionally, the EMD release profile was determined in vitro, which is known to differ from the in vivo situation. In this regard, in vitro and in vivo TGF-β1 release profiles from PLGA/CaP composites are necessary to prove the above-mentioned hypothesis.

Another reason for lack of significant difference in the amount of newly formed bone between the groups at 2 and 4 weeks could be the material used as a control. The PLGA/CaP composite material has already demonstrated excellent bone regenerative capacity in previous rat studies (Plachokova et al., In press). Therefore, it appears unlikely that an osteopromotive agent, such as TGF-β1, could increase substantially its initial effect on bone formation, especially when applied in such a low concentration (i.e. 200 ng).

The PLGA/CaP composite material is a fast-setting type of cement, which still provides sufficient time for molding and contouring in order to achieve precise adjustment to the defect or augmentation site (Ginebra et al., 1994; Kairoun et al., 1997). The initial material/bone (material/implant) contact is of utmost importance for integration of the filler material and early onset of remodeling. In case of discontinuity in the material/bone (material/implant) interface, fibrous tissue ingrowth between the filler and the bone/implant will delay bone formation and might result in failure. To estimate material/bone interface, bone contact measurements were performed. In the current study, the bone contact length for the PLGA/CaP composite increased from 2.76 to 39.63 mm during 8 weeks of implantation. Moreover, TGF-β1 loading resulted in an additional increase of the initial bone contact, since this material showed significantly higher values of contact length at 2 weeks in comparison with the unloaded material. The reason for this significantly increased bone contact in favor of TGF-β1 loaded composites appears to be unknown and will be a subject of a future investigation.

Concerning the material application method in this study, direct injection of the PLGA/
CaP composite without fixation was chosen due to the close resemblance of this mode of application to the final clinical approach. Nevertheless, it should be taken into account that lack of rigid fixation of the implants to the skull can have influenced bone formation. Rigid fixation is known to decrease movement and shear forces on the graft, thereby improving revascularization and resulting in earlier bone apposition and improved osteoconduction (La Trenta et al., 1989; Lin et al., 1990; Phillips and Rahn 1990). Finally, at two weeks of implantation, a serious inflammatory response to the PLGA/CaP material was observed. In view of this, it has to be noticed that the mechanical properties of the PLGA/CaP composite are limited. As a consequence, tight closure of the periosteum and subcutaneous tissue over the implants can have caused a high pressure resulting in the occurrence of fractures in the cement material. These fractures can be associated with the appearance of small CaP particles. Orthopedic implant studies have already highlighted that CaP particles can induce an inflammatory response (Pioletti et al., 2000; Shambbag et al., 1998; Grant and Jacobs 1994) or can alter osteoblast functions if their size is smaller than 10 μm (Puleo et al., 1991). As a result, bone formation will be delayed until the particles are completely resorbed. A study performed on calvarial rat osteoblasts confirmed the adverse effect of small CaP particles (<10 μm) on bone formation (Pioletti et al., 2000). In addition to the presence of small CaP particles, the cement setting and transformation can have contributed to the observed inflammatory response. These processes involve phase transformation of the ceramic with an associated substantial decrease in pH (pH was approximately 5.5) (Habraken et al., 2006). The increased acidity around the implant can be responsible for the appearance of inflammatory cells. All these observations are in agreement with the study of Huse et al. (Huse et al., 2004), who also found an extensive inflammatory response (Pioletti et al., 2000). In addition to the presence of small CaP particles, the cement setting and transformation can have contributed to the observed inflammatory response. These processes involve phase transformation of the ceramic with an associated substantial decrease in pH (pH was approximately 5.5) (Habraken et al., 2006). The increased acidity around the implant can be responsible for the appearance of inflammatory cells. All these observations are in agreement with the study of Huse et al. (Huse et al., 2004), who also found an extensive inflammatory response (Pioletti et al., 2000).

**Conclusion**

In summary, the injectable PLGA/CaP composite stimulated bone augmentation in a rat model. The addition of TGF-β1 to the composite significantly increased bone contact at 2 weeks and enhanced new bone formation at 8 weeks. For clinical application, mechanical properties of the injectable composite after setting have to be improved.

**Acknowledgements**

We would like to thank the following people for their contribution to this paper: Natasja van Dijk for sectioning and histology, Vincent Cuijpers for his help with the histomorphometry and Jurgen van Rens for the preparation of the TGF-β1 loaded PLGA/CaP composites.

References:

- Grant TT, Jacobs JJ. 1994, Response of three murine macrophage populations to particulate debris: bone resorption in organ cultures, J Orthop Res, 12: 720–731
- Moxham JP, Kibblewhite DJ, Bruce AG, Rigley T, Gillespy T, Lane J. 1996, Transforming growth factor-β1 in a guanidine-extracted demineralized bone matrix carrier rapidly closes a rabbit critical calvarial...
Phillips JH, Rahn BA. 1990, Fixation effects on membranous and endochondral onlay bone graft revascularization and bone deposition, Plast Reconstr Surg, 85: 891–897
Roberts AB, Sporn MB. 1993, Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta), Growth Factors, 8:1–9
Ruhe PQ, Hedberg-Dirk EL, Padron NT, Spauwen PH, Jansen JA, Mikos AG. 2006, Porous poly(DL-lactic-co-glycolic acid)/calcium phosphate cement composites for reconstruction of bone defects, Tissue Eng, 12: 789-800
Introduction

Emdogain® was introduced in 1997 as an alternative approach for periodontal regen-erated based on embryonic tooth formation [1, 2]. It is an extract of porcine enamel matrix, termed ‘enamel matrix derivate’ (EMD) and thought to induce mesenchymal cells to mimic the events that occur during root development and to stimulate regeneration of periodontal tissues, i.e. cementum, periodontal ligament and bone. The ability of EMD to enhance bone formation in a nude mouse model was defined as osteopromotive [3] and was suggested to be due to the enhancement of the osteoinductive potential of the graft material by EMD. However, it was also hypothesized that (1) EMD showed osteopromotive characteristics because of the presence of bone growth stimulating factors and (2) a threshold concentration of EMD was required to evoke an effect. Although, early immunoassay studies could not identify the presence of growth factors in EMD [4], later on, on nominal levels of Transforming Growth Factor - beta 1 (TGF-ß1) [5] as well as BMP-2 and BMP-4 were detected in an osteoinductive fraction in enamel extracts [6].

Calcium phosphate (CaP) ceramics are extensively used as bone substitute in dentistry, orthopaedics, and reconstructive surgery due to their biocompatibility and osteoconductivity. Frequently, CaP ceramics are manufactured and used in the form of granules or prefabricated (porous) blocks, which are unfortunately difficult to apply from a clinical point of view, e.g. difficulty of local maintenance of the material, lack of shaping, difficult delivery in the bone defect. These disadvantages have been overcome by the development of CaP cements, which can be injected in the defect site, shaped according to the defect dimension and set in situ. However, depending on the composition of the final set product, the in vivo resorption and tissue ingrowth can be slow [7]. An approach to overcome this limitation is the creation of a macroporous structure within the finally set CaP cement. Such a concept has recently been developed in our laboratory. PLGA microparticles were incorporated in the cement to induce macroporosity [8]. Additionally, the inclusion of degradable microparticles provided a method for sustained delivery of bioactive molecules, which enhanced the bone healing process [9, 10, 11].

In view of the above mentioned, the objective of this experiment was to investigate the bone regenerative properties of Emdogain®. We hypothesized that EMD incorporated in different concentrations into PLGA/CaP composites could enhance bone-healing due to the presence of osteoinductive growth factors in the EMD. To prove this hypothesis, PLGA/CaP composite implants and EMD incorporated PLGA/CaP composite materials were inserted in an orthotopic (cranial) and ectopic (subcutaneous) location in rats.

Materials and Methods

Preparation of microparticles

PLGA microspheres were prepared using a (w/o/w) double emulsion solvent evaporation technique. Briefly, 1.4 g of low molecular weight (LMW) PLGA was dissolved in 2 ml of dichloromethane (DCM) inside a 50 ml PP tube. 500 ml of ddH₂O (or 8.0/11.2 % BSA in ddH₂O) was added while vortexing vigorously for 1 min, subsequently adding 6 ml of a 0.3% PVA solution. Vortexing was continued for another 1 min. The content of the 50 ml tube was transferred to a stirred 1000 ml beaker and another 394 ml of 0.3% PVA was added slowly. This was directly followed by adding 400 ml of a 2% isopropyl alcohol (IPA) solution. The suspension was stirred for 1 hr. The spheres were allowed to settle for 15 min and the solution was decanted. The suspension left was centrifuged, and the clear solution at the top was decanted. 5 ml of ddH₂O was added, the spheres were washed, centrifuged and the solution was aspirated. Finally, the spheres were frozen, freeze-dried for 24 hr and stored under argon at -20°C.

Preparation of PLGA/CaP implants

Implants were made by adding low-molecular poly (D, L-lactic-co-glycolic acid) (PLGA) microspheres (Purasorb®, Purac, Gorinchem, the Netherlands) to calcium phosphate cement (Calcibon®, Biomet Merck, Darmstadt, Germany), so that a weight ratio 20/80 of PLGA/CaP cement was achieved. The chemical composition of the CaP cement was 62.5% α-TCP (alpha tri-calcium phosphate), 26.8% CaHPO₄, 8.9% CaCO₃ and 1.8% PHA (precipitated hydroxyapatite). An aqueous solution of 1 % Na₂HPO₄ was used as a liquid component with a liquid/powder ratio of 0.35 ml/g. The cement powder was sterilized by gamma radiation with 25 kGy (Isotron B.V., Ede, the Netherlands) and the cement liquid was filter-sterilized (0.2μm filter). Firstly, CaP cement powder provided with PLGA microparticles was added to a 2 ml plastic syringe. Subsequently, 1% Na₂HPO₄ was applied to the mixture and the mixture was shaken vigorously for 30 sec using a Silamat® mixing apparatus (Vivadent, Schaan, Liechtenstein). After mixing, the cement was immediately injected into a round mould to ensure a standardized shape of the samples. In this way, implants in the form of discs were created with a diameter of 8 mm and a height of 2 mm. These discs were removed from the molds after setting of the cement. The average weight of the samples was 140 mg.

Adsorption of EMD on PLGA microspheres

Thirty mg Emdogain® (BIORA AB, Malmö, Sweden) was dissolved into 800 μl of 0.1%
acetic acid and added to 1.0 g of microspheres, after which the EMD was freeze-dried onto the microspheres.

**Preparation of EMD/PLGA/CaP cement implants**

PLGA/CaP cement implants provided with EMD were made by adding a fraction of EMD adsorbed PLGA microspheres to a fraction of as-prepared PLGA microspheres and subsequent mixing of both fractions through CaP cement powder. By adding different amounts of EMD adsorbed microspheres, implants with different concentrations of EMD were obtained, i.e. 0.25 mg/implant, 0.50 mg/implant and 0.80 mg/implant. For the final shaping and setting of the implants the same procedure was followed as described above for non-loaded cement implants.

**Surgical procedure**

The loaded and unloaded composites were inserted orthotopically and ectopically in rats for 4 weeks.

**Orthotopic implants**

For the orthotopic implantation, 24 healthy skeletally mature male Wistar rats, with an average weight of 250 g, were used. National guidelines for the care and use of laboratory animals were observed. In total, 24 implants were inserted into the parietal cranial bone of the rats resulting in four different groups of rats:

1. Group A - unloaded PLGA/CaP composites (n = 6)
2. Group B - 0.25 mg EMD per implant loaded PLGA/CaP composites (n = 6)
3. Group C - 0.50 mg EMD per implant loaded PLGA/CaP composites (n = 6)
4. Group D - 0.80 mg EMD per implant loaded PLGA/CaP composites (n = 6)

For the installation of the implants, full-thickness, critical-sized cranial defects with a diameter of 8 mm were created [12]. Therefore, anesthesia was induced with 4% Isoflurane and maintained with 2% Isoflurane and 0.4% O₂ by non-rebreather mask and monitored to ensure that an appropriate level of anesthesia was achieved and maintained for surgery. The animals were premedicated by an intramuscular injection of Fentanyl (2.7 ml/kg) to reduce the operative pain, and a subcutaneous injection of Buprenorphine (150 μg/kg) was applied to reduce the postoperative pain. Before surgery, the dorsal part of the rat cranium was shaved and swabbed by iodine. A median sagittal incision extending from the nasofrontal area to the occipital protuberance was made and soft tissues were sharp dissected to visualize the cranial periosteum. The periosteum was then undermined and reflected, exposing the parietal bones. A hollow trephine bur (ACE Dental Implant System, USA) with an outer diameter of 8 mm in a dental hand piece was used to create a full-thickness defect into the dorsal part of the parietal bone. Although the defect included the sagittal suture, care was taken not to damage the dura mater or to puncture the superior sagittal sinus. After insertion of the various materials, the periosteum and overlying skin were closed in separate layers with 5-0 and 4-0 Vicryl resorbable sutures.

**Ectopic implants**

For the ectopic implantation, eight male Wistar rats of 100 g were used. Each rat received four implants subcutaneously according to a randomization scheme. In total, 32 implants were placed, as follows:

1. Group A - unloaded PLGA/CaP composites (n = 8)
2. Group B - 0.25 mg EMD per implant loaded PLGA/CaP composites (n = 8)
3. Group C - 0.50 mg EMD per implant loaded PLGA/CaP composites (n = 8)
4. Group D - 0.80 mg EMD per implant loaded PLGA/CaP composites (n = 8)

Surgery was performed under general inhalation anesthesia with a combination of 2% Isoflurane, 0.4% N₂O, and 0.4% O₂. The composites were subcutaneously implanted into the back of the animals. Four small longitudinal incisions were made through the full thickness of the skin on both sides of the vertebral column. Lateral to the incisions, a subcutaneous pocket was created using blunt dissection. Subsequently, one implant was inserted in each pocket. Finally, the skin was closed using Agraven suture material.

**Histological preparation**

The animals were euthanized using an overdose of carbon dioxide. For the orthotopic implants, the skin was dissected and the defect sites were removed along with the surrounding bone and soft tissues, and fixed in 10% neutral formalin for one week. For the orthotopic implants, the skin was dissected and the defect sites were removed along with the surrounding bone and soft tissues, and fixed in 10% neutral formalin for one week. The specimens were left undecalcified and were embedded in polymethylmethacrylate (PMMA). After polymerization, sections were prepared using a modified diamond-blade sawing microtome technique (Leica Microsystems GmbH, Wetzlar, Germany). The sections were 10 μm thick and were stained with methylene blue and basic fuchsin.

For the ectopic study, implants with their surrounding tissue were retrieved, fixed in 10% neutral formalin for one week, and embedded in PMMA. Further, the same procedure for sawing and staining was carried out as described above. The light microscopical evaluation of all samples (both from the orthotopic and ectopic
study) was done using an optical microscope (Leica BW, Rijswijk, the Netherlands) and consisted of a complete morphological description of the tissue response to the different implants.

**Histomorphometry**

Histomorphometrical analysis was performed only for the cranial implants. Measurements were done on three digitalized sections per cranial specimen to quantify bone ingrowth within the defect. For this purpose, a Leica Qwin Proimage analysis system (Leica BV, Rijswijk, the Netherlands) was used and sections were digitized at low magnification (2.5x). The newly formed bone, as defined by its woven structure and location, was marked in an interactive manner and the computer measured its length (BL) in mm. Another parameter to determine was the total length of the defect, i.e. the area (in mm) between the defect borders that has to be filled with newly formed bone (Figure 4A). From these two data, the length of the bone ingrowth in percentage (%) was calculated. Presented results were based on the average of these measurements.

**In vitro EMD release test**

To determine the release of EMD out of the PLGA/CaP implants a release test was done. Therefore, a sample of each EMD loaded implant group was put into a 15 ml tube with 1 ml of Milli Q and incubated in a water bath (37°C) to mimic the conditions in the rat body. After 1, 3, 5 hours and 1, 2, 4, 7, 14, and 28 days, 1 ml sample of Milli Q was taken from the tubes and replaced by 1 ml fresh Milli Q. The sample was put into a 15 ml tube and freeze-dried for 1 day. Subsequently, it was dissolved in 200 µl of MilliQ. In this way, a 5 times higher concentration of EMD was achieved, which facilitated final analysis. To determine the drug release per week and cumulative release, the concentration of the release medium was measured by HPLC (high performance liquid chromatography) using a RP (reversed- phase) - column (Atlantis®, Waters corp., Milford, MA, USA). A 30/70 mixture of acetonitril/water was used with phase containing 10% 0.1 M formic acid. Before each analysis, samples were filtered with Acrodisk® filters.

**Statistical analysis**

For the statistical analysis, GraphPad InStat program (GraphPad Software, San Diego, CA.) was used. One-way Analysis of Variance (ANOVA) was performed on the data obtained from the histomorphometric analyses. ANOVA assumes that the data are sampled from populations with identical standard deviations. This assumption was tested using the method of Bartlett. Further, ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption was tested using the method Kolmogorov and Smirnov. In addition, a Tukey-Kramer Multiple Comparisons Test was done. Differences were considered to be significant when p < 0.05.

**Results**

All animals had an uneventful recovery. Their weight remained stable and increased during the implantation period. Further, no infection occurred during that time.

**Descriptive histology**

**Cranial implants**

Light microscopic examination after 4 weeks showed the presence of newly formed bone and no complete closure of the defects in all experimental groups. The bone ingrowth was initiated mainly from the dura side and defect edges, whereas no bone formation from the periosteal side was observed. Only a layer of connective tissue was present on the top of the composites that penetrated into the macropores as created by the degraded PLGA particles close to the surface. There was no difference in thickness and vascularization of this connective tissue layer between the groups.

Newly formed bone was most abundant for unloaded implants and implants loaded with 0.50 mg EMD. Cranial defects provided with 0.25 mg EMD loaded implants showed minor bone ingrowth. PLGA/CaP implants with 0.80 mg EMD induced bone formation comparable with the 0.25 mg EMD implants (Figure 1). The newly formed bone was in direct contact with the surface of both loaded and unloaded composites without any fibrous tissue interface (Figure 2A). Evidently, bone apposition was guided over the surface of the PLGA/CaP implants (Figure 2B).

![Figure 1: Light micrographs of PMMA embedded specimens showing bone ingrowth into cranial defects (0.6x magnification)](image1)

**Figure 1:** Light micrographs of PMMA embedded specimens showing bone ingrowth into cranial defects (0.6x magnification)

A) unloaded implants
B) 0.25 mg EMD loaded implants
C) 0.50 mg EMD loaded implants
D) 0.80 mg EMD loaded implants

![Figure 2: Histological sections of PMMA embedded specimens. Guided bone formation is visible with direct contact cement-newly formed bone without any sign of a fibrous tissue interface](image2)

**Figure 2:** Histological sections of PMMA embedded specimens. Guided bone formation is visible with direct contact cement-newly formed bone without any sign of a fibrous tissue interface

A) at the side of the dura: 40 x magnification
B) within a crack of an implant: 20 x magnification
At higher magnification, the newly formed bone revealed thin trabeculae and large marrow spaces filled with fat tissue and haematopoietic cells. Newly formed bone had a woven structure without preferred orientation of the collagen fibers in the bone matrix and an abundance of osteoblasts and small blood vessels. Occasionally, some multinucleated inflammatory-like cells were seen in contact with the cement, but they did not occur specifically for loaded or unloaded implants.

All implants were maintained in the cranial defects during the implantation time and kept their shape and stability.

PMMA samples showed complete maintenance of integrity of the undecalcified composites. They revealed very limited degradation of the PLGA microparticles, especially in the middle of the composites. At the space left by the degraded microparticles, which was mainly at the dura and periosteal surface of the implants, fibrous tissue was observed inside the resulting microporosity. In the center of the composites, no degradation of the PLGA at all was seen (Figure 3).

**Histomorphometry**

Histomorphometric measurements confirmed the subjective evaluation. Bone formation was most abundant for unloaded implants (54 % ± 15.0 bone ingrowth) and lowest for the 0.25 mg EMD implants (19 % ± 22.5 bone ingrowth). For the 0.50 mg EMD implants, the bone ingrowth was 40 % ± 23.6, whereas for the 0.80 mg implants, it was 26 % ± 17.6. Statistical testing showed no significant differences (p > 0.05) in the amount of the newly formed bone among the EMD groups. There was a statistically significant difference (p < 0.05) only between the unloaded implants and those loaded with 0.25 mg EMD in favour of the unloaded implants (Figure 4B).

**Ectopic implantation**

Light microscopic analysis of the subcutaneous sections revealed no bone formation in all groups after an implantation period of 4 weeks. Only a layer of highly vascularized loose connective tissue was present around the composites, which penetrated into the macropores of the PLGA particles most close to the implant surface (Figure 5). There was no apparent difference in the thickness of this fibrous capsule or in vascularization among the various implant groups. Occasionally, some multinucleated inflammatory-like cells were observed at the surface of all implant groups.

**In vitro EMD release study**

The results of the in vitro EMD kinetics are depicted in Figure 6. An initial burst release of about 10% of the total dose of EMD was observed during the 1, 3 and 5 hours for all EMD loaded implant groups. Subsequently, a sustained release profile was observed during the 1st week of incubation. Thereafter, the release increased rapidly (Fig 6A) and was calculated to be about 35% per week. At day twenty-eight, 60% of the total amount of EMD loaded in the PLGA/CaP implants was released in the medium (Fig 6B).
Discussion

The present study failed to support the hypothesis that EMD can enhance bone regeneration when applied in combination with an osteoconductive material orthotopically and induces bone formation ectopically. On the contrary, less bone formation was observed compared with the PLGA/CaP composites alone in the critical-sized cranial defects. Nevertheless, 0.50 mg EMD loaded implants tended to result in more bone ingrowth than 0.25 mg and 0.80 mg EMD loaded implants. This finding is in agreement with the in vitro study of Yoneda et al. 2003 [13], in which the effect of EMD on osteoblastic cells was evaluated. They demonstrated that EMD affected the cell proliferation, ALP activity and mineralized nodule formation in a concentration-dependent manner, whereas the most favorable concentration of EMD was reported to be 0.50 mg. On the other hand, in the in vivo part of the same study, in which EMD-loaded collagen pellets were inserted into 3.8 mm full thickness cranial defects for an implantation period of 2 weeks, the released EMD stimulated new bone formation, which does not corroborate our observations. This discrepancy can be due to the fact that in the in vivo experiment of Yoneda et al. smaller defects (3.8 mm in diameter versus 8 mm), shorter implantation periods (2 weeks versus 4 weeks), and a higher concentration of EMD (1 mg versus 0.25 mg, 0.50 mg, 0.80 mg) were used, which might have resulted in better outcomes. Moreover, a different delivery vehicle (collagen pellet versus PLGA/CaP cement) was used, and although the EMD release profile was not reported, it might also have affected the study results. Further, it has to be noticed that Yoneda et al. included an empty defect as control group, whereas we used PLGA/CaP implants as control, which have already demonstrated excellent osteogenic capacity. In our study 0.80 mg EMD loaded composites caused less bone ingrowth than the 0.50 mg EMD loaded (40% bone formation versus 27%). An explanation can be that there is an optimal dose of action of EMD, which when being exceeded or not reached results in an inhibitory effect. This was already observed in an in vitro study with EMD and PDL cells [14]. In this study, the viability of PDL cells was observed negatively affected by higher doses of EMD over time, while lower doses elicited no change when compared with control cultures.

The conclusion of the present study that EMD does not provide an additional stimulus for bone formation is supported by the findings of Donos et al. [15, 16]. In one of their studies, cranial defects with a diameter of 5 mm were treated with barrier membranes and/or EMD, and demineralised bovine bone matrix (DBBM) plus EMD versus a combination of a barrier, EMD and DBBM for 4 months. The combined use of the barriers with EMD did not significantly enhance bone healing. Therefore, the authors even suggested that the use of EMD for the purpose of generating new bone in clinical situations should be questioned. The same is true for the possible osteoinductive nature of EMD. Previous studies of Boyan et al. [3] and Yoneda et al. [13], suggested already that EMD does not have bone morphogenetic protein (BMP)–like osteoinductive activity. Our investigation confirmed this observation, since no bone formation was found when EMD composites were implanted subcutaneously for 4 weeks. Delivery systems are of great importance when growth factors are used to enhance wound healing [17, 18]. Considering our histological findings, i.e. the lack of a bone formation enhancing effect of the EMD-loaded PLGA/CaP implants, it can be assumed that the present delivery system of EMD was not appropriate. In view of this, an in vitro release study was done to obtain information about the release kinetics of EMD. Our findings correspond to the release profiles of other growth factors from the same CaP cement [9, 10]. In our study, the initial burst release of 10% was due to the release of EMD from the surface of the scaffold. Afterwards, the release was sustained until day 7, when low molecular PLGA particles started to erode. At day 14, the erosion of the PLGA particles was complete, which resulted in fast increase in the EMD release profile. After four weeks, 60% of the total amount of EMD present in the PLGA/CaP composites was released in the medium and it seemed that this increasing pattern continued. However, in vivo release patterns are known to differ from the in vitro situation with a lower release profile in vivo compared with in vitro [9, 10]. Besides that, CaP cement has been reported to exhibit a high protein binding affinity [19]. As a consequence, interaction of EMD with CaP cement can have occurred, which can have hampered additionally the final release of EMD. Therefore, it cannot be excluded that the released EMD failed to reach the dose required to evoke an effect. However, further investigations with other carrier materials or higher concentrations of EMD, as well as in vivo release assays of EMD are required to prove this hypothesis.

The current results confirmed again the excellent biocompatibility and osteoconductivity of PLGA/CaP cement [20, 21]. Although, the material was applied in a preset condition, defects with unloaded PLGA/CaP composites showed the most abundant bone formation. In all cranial specimens, a direct contact between the newly formed bone and the composites was found, whereas a few multinucleated inflammatory cells were seen on the cement surface.

Conclusion

In summary, we conclude that in the current study Emdogain® was found to lack osteoinductive properties and was not able to enhance bone healing in combination with an osteoconductive PLGA/CaP material. However, the limited release pattern of EMD for the period of 4 weeks should be taken into account. Therefore, as the exact action mechanism of EMD is still unknown, further investigations are required.
Acknowledgements

We would like the following people for their contribution to this paper: Jurgen van Rens for the preparation of the PLGA/CaP composites and the EMD-release study, and Vincent Cuijpers for his help with the histomorphometry, Natasja van Dijk for sectioning and histology.

References

Chapter 7


Chapter 8

General discussion
1. Concluding remarks

During the last decades, the improved understanding of wound healing and tissue regeneration processes together with the scientific discoveries in cellular and molecular biology have led to the development of novel multidisciplinary fields like regenerative medicine and tissue engineering. Regenerative medicine and tissue engineering employ biomimicry, since they seek to mimic the body's natural tissue formation processes to form functional living tissue. As a consequence, the concept of "biological solutions to biological problems" has emerged as a new paradigm in contemporary dentistry and medicine (Slavkin & Bartold 2006). For example, as a consequence of the development of biologically active additives, such as growth factors, platelet-rich plasma and enamel matrix derivates are currently used in clinical procedures for bone regeneration. While in their majority these biologically active additives offer improvement in clinical results, better understanding of their exact mechanism of action is still needed. In view of this, the main research outcomes of the chapters of this thesis are discussed below.

PRP

The use of PRP is a relatively new approach, which clinical efficiency is supposed to be due to the local and continuous delivery of a wide range of GFs and proteins (adhesive molecules), mimicking the needs of the physiological wound healing and reparative tissue processes. Recently, upon its development PRP has been directly introduced into the clinic as an enhancer for bone regeneration (Marx et al. 1998), which caused a big discussion among the researchers about the biology of PRP, its mode of action and relevance for clinical application (Schmitz & Hollinger 2001, Freymiller & Aghaloo 2004). Nevertheless, since then, PRP has been widely used in the dental practice for treatment of periodontal defects, sinus lift procedures, oral-maxillofacial reconstructions, alveolar ridge augmentations, implant placement and treatment of extraction sockets. Overall, in the dental literature the reported effects of PRP on bone healing in humans are contradictory. Our systematic review (Chapter 2), undertaken to address this issue, found evidence only for the use of PRP in treatment of periodontal defects. Current evidence for the beneficial effect of PRP on bone regeneration in sinus elevation procedures was considered weak. Furthermore, this systematic review revealed a serious lack of good quality clinical trials, which made it impossible to draw solid conclusions on the relevance of other PRP applications.

In an attempt to give some more insight into the mode of action of PRP, its early and late effects on bone healing were studied in a rat model (Chapter 3 and Chapter 4). Syngenegetic animals were chosen for these experiments to meet the main requirement of PRP as an autologous product. Further, rat PRP as prepared had a 3-fold increase of platelets above their base-line number in the whole rat blood, which is in correspondence with the concentration of platelets obtained by one of the FDA approved devices (3i PCCS) (Marx 2004). Our results showed that at both early and late time points PRP failed to enhance bone regeneration in combination with an osteoconductive material. In addition, no difference between PRP liquid and PRP gel on bone formation was observed, except for the beneficial effect of the gel on the ease of handling of the graft. To elucidate further the bone regenerative effect of PRP and to provide an explanation for the discrepancy in the literature about its impact on bone healing, it was decided to examine and compare some of the most studied experimental PRP formulations (rat, goat and human) (Chapter 5). Our findings showed that rat and goat PRP had no effect on bone formation neither at early nor at late time points in the used immunodeficient rat model. In contrast, human PRP was able to enhance the initial osteogenic capacity of human bone graft material, and its addition to an autogenous bone graft appeared to be more beneficial than to a synthetic bone substitute. These findings corroborate the hypothesis of the mode of action of PRP and confirmed the results of our previous studies.

TGF-β1

The importance of TGF-β1 in bone development and homeostasis has been extensively demonstrated both in vitro and in vivo with strong evidence for profound effects on bone formation, bone resorption, and the interplay between these two processes. However, results obtained from different studies are often not in line with and even contradictory to each other. In this research (Chapter 6), the bone regenerative effect of TGF-β1 in combination with an osteoconductive material was examined. TGF-β1 applied in a low concentration was able to increase the initial bone contact and to enhance bone augmentation at later time points. Several questions arose from our findings. It remained unclear why TGF-β1 failed to stimulate the early amount of bone formation, but at the same time increased the initial bone contact. Additional research is required to elucidate the observed effects of TGF-β1.

EMD

In 1997 Hammarström introduced an alternative approach for periodontal regeneration based on biomimicry (Hammarstrom 1997). This approach uses EMD to mimic the events that occur during root development and thereby to stimulate periodontal regeneration. Results from in vitro and in vivo studies have shown that EMD is able to induce regeneration of cementum, periodontal ligament and alveolar bone. However, the specific characteristic of EMD regarding just bone formation is still ambiguous. In this research (Chapter 7), the bone regenerative properties of EMD were investigated...
in combination with an osteoconductive material. Our findings revealed that EMD was neither osteoinductive, nor able to enhance bone healing. The lack of osteoinductivity of EMD has already been reported, but in the majority of the studies, a stimulatory effect of EMD on bone growth has been demonstrated. Therefore, the outcomes of our study must be interpreted within the limitations of the study model. Further studies with other carrier materials and higher concentrations of EMD are necessary to corroborate these results.

2. Future perspectives

With an increasing number of older people and an increasing life expectancy worldwide, there is an increasing clinical need for effective and minimal invasive bone regeneration treatment options. The “holy grail” of bone regeneration is the development of “smart” materials that do not require additional biologically active factors. These materials are alleged to exactly mimic the natural bone environment, serving as a scaffold for the orchestrated activation of local cellular processes.

PRP has appeared as a promising innovation for stimulation and acceleration of the bone healing process. However, the exact mechanisms by which PRP enhances initial bone healing has still to be fully understood. Therefore, suitable animal models/preparations are necessary to elucidate the effects of PRP. One of the aspects for future research must be the standardization of the animal PRP preparations and their complete adjustment to the human PRP in view of procurement technique, concentration of platelets and increase above the platelets base number achieved, level of GFs, accelerators. In this respect goat PRP appears to be a suitable model, due to the comparable number of platelets between the human and goat blood. However, further research is required for example to elucidate the discrepancy in the data about the exact number of platelets in the goat blood.

In addition, the combination of PRP with different kind of synthetic bone substitutes must be further explored. Significant advance in the promotion of bone healing may be expected by the development of an appropriate scaffold for PRP, which on one hand, approximates the natural extracellular matrix and on the other, allows delivery of the GFs stored in PRP in a specific spatial and temporal sequence. This may not only enhance bone formation, but also enable engineering of large volumes of functional bone tissue in the future. Finally, well-designed controlled clinical trials are necessary to demonstrate the bone regenerative effect of PRP and to provide solid evidence for its clinical application.

With regards to TGF-β1, future research is required to reveal which is the optimal dose of TGF-β1 to enhance bone healing and which is its optimal carrier for treatment of large bone defects, so that it can reach clinical application.

In view of EMD, it appears reasonable to assume that the matrix-cell principle of Endogain® can also be applied in other clinical situations, besides treatment of periodontal defects. The use of an insoluble matrix that initiates a series of cellular events may be especially favorable, since cells involved will then provide the GFs and tissue components necessary for regeneration.
References:

Chapter 9
Summary and Address to the Aims
The present research is focused on the application of different biologically active additives for bone tissue regeneration. The aim was to acquire more information about the efficacy of their bone regenerative properties and relevance for clinical application.

In Chapter 1 a brief overview of different approaches for bone tissue regeneration is presented, while the emphasis is placed upon biologically active additives currently used for this purpose.

The aim of Chapter 2 was to summarize data available in the literature and to provide evidence for clinical application of platelet rich plasma (PRP) in oral maxillo-facial bone regeneration. For this purpose, up to June 2006, MEDLINE and Cochrane databases were explored with different combinations of three search terms: “PRP”, “bone regeneration”, “dentistry” and their synonyms. Inclusion criteria were: human controlled clinical trials designed to treat maxillo-facial bony defects with application of PRP (test) or without PRP (control), including at least 5 patients with a follow-up period more than 3 months and using clinical assessment, radiography, histology and /or histomorphometry as evaluation. Literature search, selection of eligible articles, and data extraction were carried out independently by two readers. The literature search revealed 108 references, of which 17 were selected for further analysis. Finally, 9 articles fulfilling the inclusion criteria, were selected to review systematically. Due to the substantial heterogeneity of the studies it was not possible to statistically analyse the data. An attempt was made to compare results from studies that used similar outcome measures by calculating and adding Confidence Intervals to the data presented in the original papers. Differences in treatment effects for periodontal defects in terms of clinical attachment level (CAL) were significant (ranging from 0.8 to 3.2 mm). The reported effects of PRP were almost completely closed. It was concluded that rat PRP had no effect on the early stages of bone healing in addition to an osteoconductive material. Dense HA/ß-TCP particles with size 300-500 μm and ratio 60/40 showed a beneficial effect on bone formation during the first and second week in non-critical-sized cranial defects in rats. A 6.2 mm cranial defect turned out not to be a critical-sized defect in rats.

Chapter 4 deals with the late effect of platelet-rich plasma on bone regeneration. Cranial defects, 6.2 mm in diameter, were filled with HA/ß-TCP particles, HA/ß-TCP particles combined with PRP and HA/ß-TCP particles combined with PRP gel, where some were left empty as a control. After four weeks of implantation histological, histomorphometrical and μCT analyses revealed no difference in new bone formation among the groups. Further, no additional effect of PRP gel in comparison to PRP liquid was detected, except for the increased handling capacity of the graft. These findings suggested that PRP had no positive effect on bone formation in addition to an osteoconductive material after an implantation period of four weeks. On the other hand, also no negative effect was seen, neither PRP nor HA/ß-TCP hampered bone ingrowth into the defects.

The aim of Chapter 5 was to examine and compare the bone regenerative effect of PRPs of different species (rat, goat, human). In addition, osteogenic capacity of human bone graft (HB) vs. HB combined with human PRP (HB+PRP) and HB+PRP vs. synthetic hydroxyapatite-tricalcium phosphate bone substitute combined with hPRP (HBTCP+hPRP) was studied. For this purpose, 72 implants, divided in six groups (n = 6) were inserted in critical-sized cranial defects (8 mm) of immunodeficient rats. After two and four weeks, descriptive and quantitative histological, and μCT analyses were performed on the retrieved specimens. Results showed that rat and goat PRP combined with HA/TCP did not enhance bone regeneration compared performed on the raw data obtained from μCT analysis. Light microscopic evaluation showed no effect of PRP on bone formation, neither during the first week, nor during the second week. Further, no effect of PRP on neovascularisation was observed, since no difference in the number of the new blood vessels was subjectively detected in the trichrome stained samples. On the other hand, the trichrome staining indicated an increase in bone maturity after two weeks in both groups with particles. In view of the dense HA/ß-TCP particles (60/40), hematoxylin-eosin and methylene blue/basic fuchsin stained slides revealed their osteoconductive nature. The quantitative μCT analysis demonstrated a statistically significant difference in new bone formation between the empty defects and the defects filled with particles after one week of implantation, but there was no difference between the non-PRP and PRP groups. At the second week no difference among all groups was observed, while even the non-filled control defects were almost completely closed. It was concluded that rat PRP had no effect on the early stages of bone healing in addition to an osteoconductive material. Dense HA/ß-TCP particles with size 300-500 μm and ratio 60/40 showed a beneficial effect on bone formation during the first and second week in non-critical-sized cranial defects in rats. A 6.2 mm cranial defect turned out not to be a critical-sized defect in rats.
to HA/TCP. In contrast, human PRP combined with HA/TCP resulted in significantly increased bone fill compared to HA/TCP alone. Similarly, the addition of human PRP to human bone graft increased significantly the amount of newly formed bone after two weeks. Evidently, HB+hPRP demonstrated enhanced bone healing compared to HA/TCP+hPRP. It was concluded that rat and goat PRP had no effect on bone formation. Human PRP improved the initial osteogenic response of human bone graft. Human PRP combined with human bone graft had better osteogenic capacity than human PRP combined with synthetic bone substitute.

Chapter 6 deals with the bone augmentation properties of Transforming Growth Factor β1 (TGF-β1; 200 ng) incorporated in an injectable poly(lactic-glycolic acid)/calcium phosphate PLGA/CaP (20/80) composite. For this purpose, PLGA/CaP composites (control) and PLGA/CaP composites loaded with TGF-β1 (test group) were injected on top of the skulls of 24 Wistar rats. Each rat received two materials from the same experimental group, and in total 48 implants were placed (n = 8). After two, four, and eight weeks the results were evaluated histologically and histomorphometrically. The contact length between the implants and newly formed bone increased in time, and it was significantly higher for the TGF-β1 composites after two weeks. Also, bone formation was significantly higher for the TGF-β1 composites (18.5% ± 3) compared to controls (7.21% ± 5) after eight weeks of implantation. The immunohistochemical staining demonstrated massive inflammatory infiltrate in both groups, particularly at two weeks, which decreased substantially at four and eight weeks. It was concluded that the injectable PLGA/CaP composite stimulated bone augmentation in a rat model. The addition of TGF-β1 to the composite significantly increased bone contact at two weeks and enhanced new bone formation at eight weeks.

The aim of Chapter 7 was to evaluate the bone regenerative properties of Emdogain® (EMD) in osseous and non-osseous sites. For the orthotopic study, unloaded PLGA/CaP implants, and PLGA/CaP implants loaded with different concentrations of EMD: 0.25, 0.50 and 0.80 mg/implant were inserted into cranial defects of 24 rats. The implantation time was four weeks. For the ectopic study, 32 implants were placed subcutaneously. The same study period and groups as in the orthotopic study were used. Methods of evaluation consisted of descriptive histology, histomorphometry, and an in vitro EMD release study. In the orthotopic study, new bone formation was most abundant in unloaded implants followed by 0.50 mg EMD composites. Histomorphometric measurements showed 54 % ± 15.0 bone ingrowth for unloaded, 19 % ± 22.5 for 0.25 mg, 40 % ± 23.6 for 0.50 mg and 26 % ± 17.6 for 0.80 mg EMD composites. Light microscopic analysis of the subcutaneous sections from the ectopic study revealed no bone formation in all groups after four weeks. The in vitro release study showed 60% cumulative EMD release after four weeks. It was concluded that Emdogain® was not osteoinductive, and was not able to enhance bone healing in combination with an osteoconductive material, such as PLGA/CaP cement.

The most important findings of this thesis are discussed in Chapter 8 and directions for future studies are given.
Chapter 10

Samenvatting en evaluatie van de doelstellingen
Het onderzoek beschreven in dit proefschrift is gericht op de toepassing van verschillende biologisch actieve toevoegingen voor botregeneratie. Het doel was meer informatie te verkrijgen over de werkzaamheid, de botregeneratieve eigenschappen en de relevantie voor klinische toepassing van deze toevoegingen.

In hoofdstuk 1 wordt een overzicht gegeven van verschillende aanpakken om botregeneratie te bewerkstelligen. In dit overzicht ligt de nadruk op biologisch actieve toevoegingen, welke momenteel gebruikt worden voor dit doel.

Het doel van hoofdstuk 2 was het samenvatten van beschikbare gegevens uit de literatuur en daarmee bewijs leveren voor het nut van klinische toepassing van platelet-rich plasma (PRP) ten behoeve van botregeneratie in het orale en maxillofaciale gebied. Daartoe werden databases van MEDLINE en Cochrane geraadpleegd (tijdspanne tot juni 2006) middels verschillende combinaties van drie zoektermen: ‘PRP’, ‘bone regeneration’ en ‘dentistry’ (en hun synoniemen). De ‘hits’ uit de literatuurraadpleging dienden te voldoen aan de volgende criteria: humane klinische studies gericht op de behandeling van maxillofaciale botdefecten, waarbij gebruik gemaakt wordt van PRP (test) of niet (controle), met minimaal 5 patiënten en een follow-up van minimaal 3 maanden, gebruikmakend van de volgende evaluatietechnieken: klinische beoordeling, radiografie, histologie en/of histomorfometrie. De literatuurraadpleging, selectie van geschikte wetenschappelijke artikelen en data extractie werd onafhankelijk uitgevoerd door 2 personen. De literatuurraadpleging leverde 108 ‘hits’ op, waarvan er initieel 17 werden geselecteerd. Na controle op vereiste criteria, werden uiteindelijk 9 wetenschappelijke artikelen geselecteerd voor een systematische review. Gezien de grote verschillen in opzet van de studies was het onmogelijk de data statistisch te analyseren. Derhalve werd getracht de resultaten te vergelijken van studies, waarin dezelfde parameters gebruikt werden middels de berekenen en toevoegen van betrouwbaarheidsintervallen aan de hand van de originele data van de studies. Significante verschillen werden gevonden voor behandelingseffecten voor parodontale defecten, meer specifiek voor clinical attachment level (CAL; range 0.8 – 3.2 mm). De beschreven effecten van PRP voor sinus elevatie (vergeleken met de controles) waren minder dan 10%. De conclusie van de systematische review was dat bewijs was geleverd voor positieve effecten van PRP voor de behandeling van parodontale defecten. Voor positieve effecten van PRP voor sinus elevatie was het bewijs slechts zwak. Verdere conclusies over het gebruik van PRP voor andere toepassingen in de tandheelkunde konden niet worden getrokken.

**Hoofdstuk 3** beschrijft de vroege effecten van PRP op botregeneratie. Deze vroege effecten werden onderzocht door PRP wel of niet toe te voegen aan bi-fasische keramische partikels (hydroxyapatiet/β-tricalciumfosfaat; HA/β-TCP in de verhouding 60/40) in een diermodel met craniale defecten in ratten met een diameter van 6.2 mm. De gemaakte defecten werden gevuld met enkel HA/β-TCP of met de combinatie van HA/β-TCP met PRP gel. Daarnaast werden enkele defecten onbehandeld gelaten ter controle. Op een en twee weken na operatie werden de defecten (inclusief omliggend weefsel) her- nomen voor evaluatie middels lichtmicroscopie (histologische kleuringen: hematoxylin-eosin, trichrome en basische fuchsine-methyleen blauw) en micro-CT op botformatie en neovascularisatie. Een variatie-analyse (ANOVA) werd uitgevoerd op de ruwe data van de micro-CT analyse. Lichtmicroscopisch werd geen effect waargenomen van PRP op botformatie voor beide implantatie-periodes. Daarnaast werden ook geen effecten van PRP op neovascularisatie waargenomen, aangezien geen verschillen in aantal nieuwe bloedvaten werden gezien tussen de verschillende experimentele groepen (histologische, trichrome-gekleurde coupes). Echter, trichrome-gekleurde coupes verkonden wel een verhoogde botweefsel maturatie na twee weken in beide groepen met keramische partikels. Zowel hematoxylin-eosin als basische fuchsine/methyleen blauw kleuring van de histologische coupes liet duidelijk de osteoconductieve eigenschappen van de keramische partikels zien. De kwantitatieve micro-CT analyse leverde een statistisch significant verschil op v.w.b. de formatie van nieuw botweefsel op één week tussen lege defecten en defecten gevuld met keramische partikels, waarbij opgemerkt dient te worden dat de toevoeging van PRP geen verschil maakte. Na een implantatieperiode van twee weken werd geen significant verschil gevonden tussen de verschillende groepen en zelfs de lege defecten waren bijna geheel geregenerieerd. De conclusie van deze studie was dat PRP geen additionele effect heeft op de initiële genezing van botweefsel boven het gebruik een osteoconductief materiaal. Keramische partikels met een grootte van 300-500 µm hebben een positief effect op botformatie tijdens de eerste twee weken in een non-critical-sized cranial defect in ratten. Een cranial defect in ratten met een diameter van 6.2 mm is een non-critical-sized defect.

**Hoofdstuk 4** beschrijft onderzoek naar de late effecten van PRP op botregeneratie. Craniale defecten (Ø 6.2 mm) in ratten werden gevuld met HAβ-TCP partikels, al dan niet gecombineerd met PRP of PRP gel. Daarnaast werden enkele defecten onbehandeld gelaten ter controle. Na een implantatieperiode van vier weken werden geen verschillen gezien in botformatie tussen de verschillende groepen, gebruikmakend van evaluatie middels histologie, histomorfometrie en micro-CT analyse. Daarnaast bleek...
er geen effect te zijn van de vorm waarin PRP werd gebruikt (vioeistof of gel), behalve
in het licht van de verwerking van het materiaal. Deze vindingen suggereren derhalve
dat PRP geen positief effect heeft op botregeneratie in combinatie met een osteo-
conductief materiaal na een implantiatioperiode van vier weken. Opgemerkt dient te
worden dat de toepassing van PRP in combinatie met HA/β-TCP ook geen negatieve
effecten tot resultaat had.

Het doel van hoofdstuk 5 was vergelijkend onderzoek te doen naar de botregeneratieve
effecten van PRP’ van verschillende species (rat, geit en humaan). Daarnaast werd
gereken naar de osteogene capaciteit van humaan bot en de combinatie ervan met hu-
maan PRP (hPRP), alsook die van synthetisch keramiek (HA/β-TCP) en de combinatie
ervan met hPRP. Daartoe werden 72 implantaten, verdeeld over zes groepen (n = 6), ge-
plaatst in een critical-sized cranial defect (Ø 8 mm) in immunodeficiente ratten. Na twee
een vier weken werd beschrijvende en kwantitatieve histologische en micro-CT analyse
uitgevoerd. De resultaten toonden dat PRP van rat en geit in combinatie met synthetisch
keramiek de botregeneratie niet bevorderden t.o.v. enkel synthetisch keramiek. Echter,
de combinatie van hPRP met synthetisch keramiek resulteerde wel in een significante
verhoging van gevormd botweefsel in vergelijking met enkel synthetisch keramiek. In par-
allel hiervan vertoonden ook de combinatie hPRP met humaan bot een significante ver-
hoging nieuw gevormd botweefsel na twee weken. Bovendien was de botvorming hoger
voor hPRP gecombineerd met humaan bot dan met synthetisch keramiek. Concluderend
toonde deze studie dat PRP van rat en geit originie geen effect heeft op botvorming. Hu-
maan PRP bezit wel de capaciteit om de initiële botrespons in combinatie met humaan
bot te verbeteren. Verder heeft de combinatie van hPRP met humaan bot een betere
osteogene capaciteit dan die met synthetisch keramiek.

Hoofdstuk 6 beschrijft de botvermeerderings-eigenschappen van transforming growth
factor β1 (TGF-β1; 200 ng), geïncorporeerd in een injecteerbaar poly(lactic-glycolic)
acid/calciumfosfaat (PLGA/CaP; 20/80) composiet. Ongeladen controle composiet en
TGF-β1 geladen composiet werd geïnjecteerd bovenop de schedel van 24 Wistar rat-
ten. Bij iedere rat werden twee composieten van dezelfde experimentele groep ge-
plaatst, waardoor in totaal 48 implantaten (n = 8) werden gebruikt. Histologische en
histomorfometrische evaluatie vond plaats na implantatieperiodes van twee, vier en
acht weken. De contactlengte tussen composiet en nieuw gevormd botweefsel nam
toe met de tijd, waarbij deze lengte significant hoger was voor TGF-β1 geladen com-
posiet na twee weken. Bovendien was ook de botvorming hoger voor TGF-β1 geladen
composiet (18.5% ± 3) t.o.v. ongeladen controle composiet (7.2% ± 5) na acht weken. Immuno-
histochemische kleuringen toonden een ontstekingsinflaan blinde groepen
voor en na weken, waarna de ontstekingsreactie verminderde met de tijd. De con-
clusie van deze studie was dat injecteerbaar PLGA/CaP composiet botvermeerdering
stimuleert in een ratten model. De toevoeging van TGF-β1 zorgt voor een significante
verhoging van botcontact op twee weken en botformatie op acht weken.

Het doel van hoofdstuk 7 was de botregeneratieve eigenschappen van Emdogain® te
evaluieren, zowel binnen (orthotopisch) als buiten (ectopisch) een botomgeving. Voor
de orthotopische evaluatie werden ongeladen en Emdogain®-geladen (0.25, 0.50 en
0.80 mg/implantaat) PLGA/CaP implantaten geplaatst in cranial defecten in 24 rat-
ten voor een periode van vier weken. Ectopisch werden 32 implantaten subcutaan
gelopen voor een periode van vier weken. De evaluatie bestond uit beschrijvende
histologie, histomorphometrie en een in vitro Emdogain® release experiment. In een
orthotopische omgeving vertoonden ongeladen implantaten de meeste botvorming,
gevolgde door 0.50 mg Emdogain®-geladen implantaten. Histomorfometrie toonde een
botingroei van 54 ± 15.0%, 19 ± 22.5%, 40 ± 23.6% en 26 ± 17.6% voor respectievelijk
ongeladen en 0.25, 0.50 en 0.80 mg Emdogain®-geladen implantaten. In geen enkele
de van de ectopische implantaten werd lichtmicroscopisch botformatie aangetroffen.
De in vitro release studie liet een cumulatieve Emdogain® release van 60% zien in vier
weken. De conclusie van deze studie was dat Emdogain® niet osteoinductief is en niet
in staat is om de botvorming te verbeteren in combinatie met een osteoconductief
materiaal als PLGA/CaP.

De belangrijkste vindingen van het onderzoek beschreven in dit proefschrift worden be-
discussieerd in hoofdstuk 8, waarin tevens wordt aangegeven welk toekomstig onder-
zoek belangrijk is op dit gebied.
Publicaties gerelateerd aan dit proefschrift:


6. Plachokova AS, van den Dolder J, van den Beucken JJP, Jansen JA. Bone regenerative properties of rat, goat and human PRP. Bone; submitted

Presentaties:
1. Plachokova AS, van den Dolder J, Stoelinga PJ, Jansen JA. The bone regenerative effect of platelet-rich plasma in combination with an osteoconductive material in rat cranial defects, 8th Annual Tissue Engineering Society International (TESI) Conference & Exposition (October 22-25, 2005), Shanghai, China

2. Plachokova AS, van den Dolder J, Jansen JA. The Bone Regenerative Properties of Emdogain®, IADR/AADR/CADR 85th General Session and Exhibition (March 21-24, 2007), New Orleans, USA- finalist for Young Investigator’s Award in the Implantology Research Group


Chapter 11
Acknowledgements
I find it difficult to consider this thesis as a personal achievement, rather I look at it as the outcome of mutual efforts of many people who supported me unconditionally through all these years. I owe them a lot, and hope to have proven that it has been worth giving me their confidence, time, and expertise. Although it is not possible to mention everyone by name, I would like specifically to acknowledge the following people.

First and foremost I would like to express my deepest gratitude to my supervisor Prof. dr. John Jansen. Dear John, thank you for the unique opportunity that you gave me to conduct research in your department and, most of all, for your confidence! It has been a challenge for me, an ordinary dentist by background, to try to attain the high scientific level that you have set in your department. Your expertise not only as a scientist, but also as a dentist convinced me that it is possible to be a researcher and a clinician at the same time. I have learned a lot from you and there is still a lot to be learned. Your extensive knowledge and creativity have always been my inspiration. I highly appreciate your perfectionism and willingness to give the best and to expect the same from me and the rest. I can’t stop admiring your working enthusiasm, your availability twenty-four hours a day, constantly willing to help and to solve problems. Your complete dedication and commitment to research and to your people, and your modest personality are remarkable. You gained my deepest respect and admiration!

I am deeply grateful to my direct supervisor dr. Juliette van den Dolder. Dear Juliette, you guided my first steps in research and helped me to develop my own ideas. It was not always easy for you, having in mind how stubborn I can be sometimes. However, you always encouraged me, and sometimes had even more confidence in me than I had in myself. Besides being my supervisor, you became also my friend. Thank you for your scientific and personal guidance, for your inspiring optimism, without which I wouldn’t have been able to succeed!

I would like to express my appreciation to my other supervisor, dr. Jeroen van den Beuckен. Dear Jeroen, although you have joined me in my research during the last phase of my study, you have played an important part in it. Your expertise and detailed attention to every word and every concept contributed greatly to improve the quality of this thesis. I highly appreciate your willingness to help, and to constantly give the best and to expect the same from me in return. I hope that we will continue to work together in the same encouraging atmosphere. Thank you!

I would like to express my gratitude to Prof. dr. Nico Creugers. Dear Nico, it has been a great pleasure for me to work with you, both professionally and personally! Thank you for giving me a broad scientific view and making me think differently!

I would like to acknowledge all the people from the Animal Facility (CDL) Radboud University Nijmegen, who helped with my experiments. My special thanks go to Gert, Ilona, Debby and Brenda, without whom I wouldn’t have been able to carry out my animal studies.

I acknowledge Cees Verhagen from Sanquin Bloedbank, Nijmegen for providing me with human and goat PRP, and Dolf Lubbers, department of Hematology, UMCN, for counting the platelets.

Ik ben mijn collega’s van de Afdeling Biomaterialen en Parodontologie van het UMC St. Radboud zeer erkentelijk voor de geweldige tijd die ik op de afdeling mocht hebben. Hartelijk dank dat jullie mij vanaf het eerste begin accepteerden als één van jullie. Ik heb me al die tijd onderdeel van jullie team gevoeld, ondanks het gegeven dat ik vaak kwam én ging. Met enkel van jullie heb ik een diep gekoesterde vriendschap gesloten. Eens zei één van jullie mij dat ik als “half Nederlands” werd beschouwd, hetgeen mij bijzonder raakte. Ik voel me erg thuis in Nederland, Nijmegen en de afdeling. Met naam en toenaam wil ik jullie bedanken voor de vriendschap, het gezelschap en de behulpzaamheid: Afsheen, Anja, Anne, Corrine, Daniel, Dennis, Dimitris, Esther, Fang Yang, Frank, George, Hongbing, Joop, Juliet, Jurgen, Kim, Lise, Manal, Marthijn, Mareijke, Meike, Natasja, Nelleke, Olga, Quinet, Ruud, Ruediger, Sander, Vincent, Walter, Weibo, Wouter, Yongang en Xuechao. Zeer in het bijzonder wil ik Esther en Sander, mijn paranimfen, bedanken. Veel dank voor jullie gezelschap en heerlijke gevoel voor humor, dat ik intens bewonder! Met de hulp ontvangen van Anja prijs ik me zeer gelukkig. Niet alleen in het laboratorium, maar ook in het dagelijkse leven en bij persoonlijke zaken was zij er altijd om naar me te luisteren en te helpen. Ook ben ik dank verschuldigd aan Natasja voor de vele uren die zij heeft besteed aan het prepareren van de histologische coupes. Speciale dank ook voor Vincent voor het uitvoeren van de micro-CT scans en voor de tekeningen die hij voor mij maakte.

A special gratitude to all the people from the department of Oral Function. During my research I had the chance to get to know you better and we have spent some unforgettable times together. Thank you Anneloes, Arie, Celeste, Chen, Dick, Toa, and Wietske in particular!

Last, but not least I want to express my deepest gratitude to my whole family and especially to my husband, my son and my mother. Without their unconditional love, support and understanding through all these years, my PhD study wouldn’t have been possible. Мило семейство, много ви обичам всички, и се надявам, че в бъдеще сте мога да компенсирам времето, което сме прекарали разделени!
Chapter 12

Curriculum Vitae
Adelina Stefanova Plachokova was born in Sofia, Bulgaria, on 9th of July 1975. She attended the German gymnasium and simultaneously the English gymnasium in Sofia as a private student, and graduated with “cum laude” in 1994. From 1994 till 2000 she studied Dentistry at the Medical University – Sofia, Faculty of Dental Medicine (Master degree). During her study she won a DAAD (Deutsch Akademischer Austausch Dienst) award and spent a month at the University of Essen in Germany. After graduation she started a private dental practice in Sofia as a general practitioner. From 2001 till 2003 she worked as an interpreter for the German journal “Quintessenz” in Bulgaria, translating articles from German into Bulgarian. Since 2002 she has been employed as an Assistant Professor at the Department of Periodontology, Faculty of Dental Medicine - Sofia. The same year she was enrolled at the post-graduate programme in Periodontology. In 2004 she won a Huygens award and spent seven months at the department of Periodontology and Biomaterials, College of Dental Science, Radboud University Nijmegen Medical Center. During that time she started her PhD study in the department under the supervision of Prof. dr. John Jansen. Since 2001 she has been married to Nikola Damyanov and has one son.
List of publications related to the thesis


Congress presentations:


Acknowledgements for the publication of the thesis: